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Microbial Transformation of 3-Nitro-4-Hydroxybenzene Arsonic Acid (Roxarsone)

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Microbial Transformation of 3-Nitro-4-Hydroxybenzene Arsonic Acid (Roxarsone)

By

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Abstract

Land application of chicken litter from production houses that use the organoarsenical 3-nitro-4-hydroxybenzenearsonic acid (roxarsone) results in deposition of significant quantities of arsenic into United States soil each year. Used widely as a feed additive in the poultry industry, roxarsone was originally thought to be stable in the environment. However, recent evidence from our lab, as well as others, has established that this benign compound is readily transformed via several intermediates into inorganic arsenate. While microbial activity has been implicated in this process, there is no direct evidence suggesting the specific processes or the organisms involved.

Using a physiological and proteomic approach, this study demonstrates increased anaerobic growth of the arsenate respiring *Clostridium* species strain OhILAs in the presence of roxarsone using two different carbon sources, as well as provides evidence to suggest the respiratory arsenate reductase (Arr) system may be constitutively expressed. Increased growth of *Clostridium* OhILAs was coupled to a loss of roxarsone as determined by spectrophotometric assays. Similar results have been obtained with the structurally similar *ortho*-nitrophenol. Additionally, arsenate reductase activity assays of OhILAs grown in the presence of lactate alone or in the presence of lactate and either roxarsone, *ortho*-nitrophenol or sodium arsenate have suggested that the respiratory arsenate reductase is constitutively expressed.

Introduction

In 1996, Bryan Sykes and colleagues published a controversial paper in the July issue of the American Journal of Human Genetics challenging the long-held belief that most of modern man's genetic influence came from an agricultural "wave of advance" postulated by Luigi Luca Cavalli-Sforza in the 1970's. While ultimately the challenge was upheld, and genetics has continued to reveal most of our genetic ancestry is rooted in our Paleolithic hunter-gatherer ancestor's genetics, there is no question that the agricultural revolution has had, and continues to be, a great source of environmental change (Sykes, 2001). In time, forests have been felled, landscapes have been altered and animals have become domesticated for the direct benefit of man. Unseen for most of this process have been the indirect affects associated with agriculture.

Unlike our distant agricultural relatives with small-scale subsistence level operations, recent agriculture has seen the development of vast corporate farms and concentrated animal feeding operations (CAFOs). Recently, surface waters have been inundated by impacts from eroding fields, fecal contamination and the spread of agricultural chemicals such as hormones, antibiotics and pesticides. Unchecked use of agricultural compounds, as well as a lack of knowledge of how these compounds behave when released into the environment, has led to a situation where potentially dangerous compounds, such as the organoarsenical roxarsone, see common use throughout the industry. Despite mankind's technological advances and attempted withdrawal from the environment, we are still intimately connected to our environment. Ultimately, adverse impacts to our environment lead to adversely impacting human health.

One such compound that has come under recent investigation is 3-nitro-4-hydroxybenzene arsonic acid (roxarsone). Used widely as a poultry feed additive to prevent coccidiosis infections, roxarsone has been marketed under the trade names 3-Nitro, 3-Nitro-10, 3-Nitro-20, and roxarsone for use both as a coccidiostat and as a growth promoter (Abdo, 1989).

Under the premise that this compound is stable when released into the environment, chicken litter containing the compound is readily applied to fields as fertilizer; though recent evidence suggests that this compound may not be entirely stable when released (Cortinas et al., 2006) (Stolz et al., 2007). The mechanisms by which roxarsone transforms in the structurally similar compound 3-amino-4-hydroxybenzene arsonic acid (3-A-4-HBAA) and ultimately into inorganic arsenate [As(V)]] are, as of yet, largely unknown. However, there is mounting evidence that demonstrates this process does occur, and at very rapid rates (Christen, 2006).

This present study examines the microbial physiology and proteomics of *Clostridium* species strain OhILAs (Figure 1). This organism was originally isolated by Asia Dawson from the Stolz Lab (Duquesne University) based on its ability to reduce arsenate to arsenite during anaerobic respiration (Fisher et al., 2007). Interestingly, pure cultures of OhILAs grown in the presence of roxarsone are capable of transforming the compound to 3-A-4-HBAA and inorganic arsenate (Stolz et al., 2007). Using a physiological and proteomic approach, this study suggests that the nitrate group on the aromatic ring serves as the terminal electron acceptor during anaerobic respiration by OhILAs (Stolz et al., 2007).

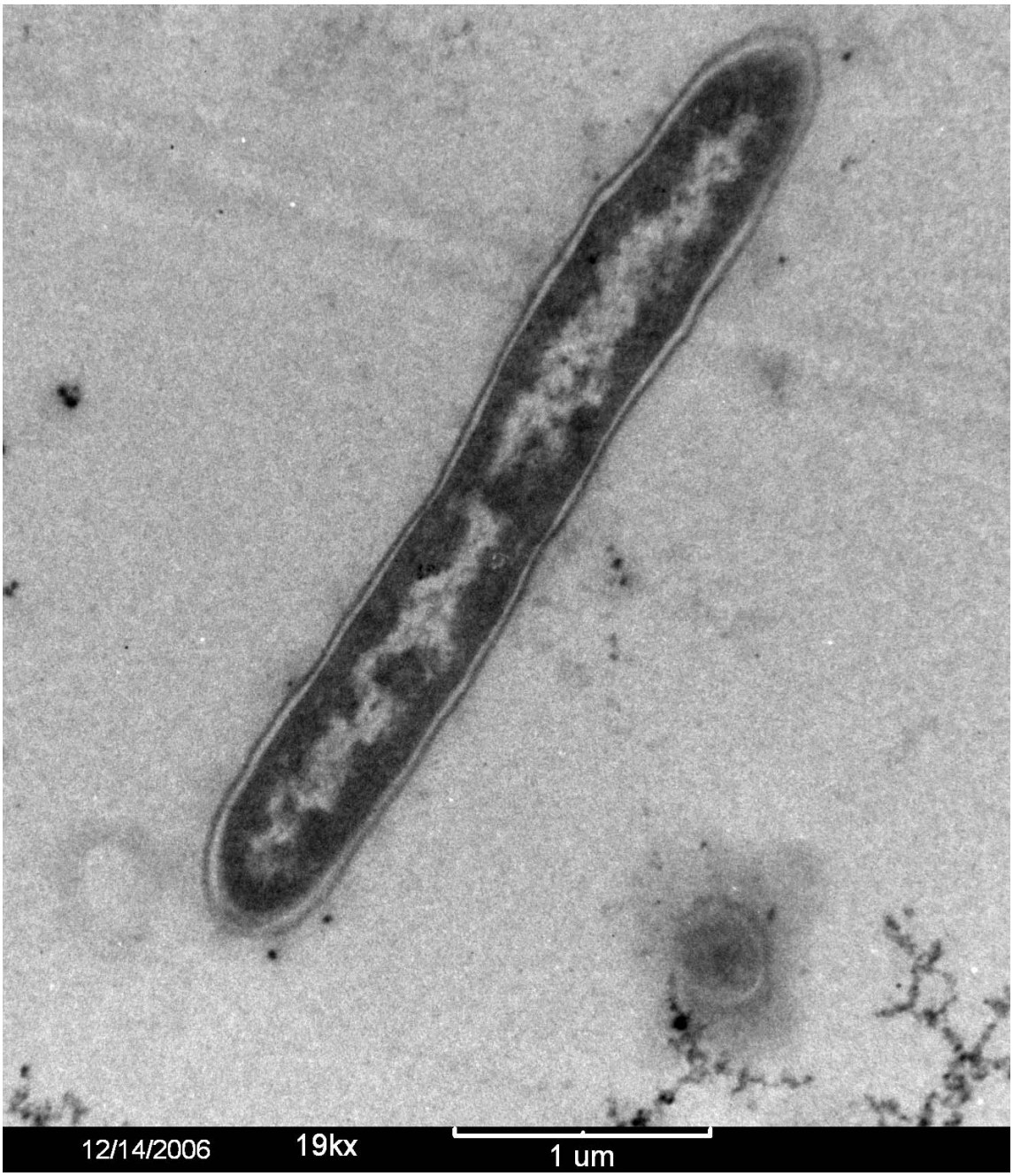


Figure 1: Transmission electron micrograph of *Clostridium* sp. strain OhILAs. Courtesy of Jonathan Franks.

Environmental Cycling of Arsenic

In 1776 a group of rebellious individuals in the American Colonies sent word that they would sever ties with their mother country, England. Sitting on the throne of England, and receiving this treasonous declaration, was King George III. One of England's longest reigning monarchs, King George was monarch for some of England's finest moments. Among these, the development of the feared British Navy, defeat of Napoleonic France and massive expansion of the British Empire. Nonetheless, he is best remembered for two exigencies: his loss of the American Colonies and his five bouts of madness. While the loss of the colonies has been the subject of much debate, and countless theses in history, for the present purpose it is more interesting to note historians widely agree the most likely explanation for his madness was a genetic metabolic disturbance known as porphyria. What is not understood is why these attacks were so severe, and lasted for very long durations (Cox et al., 2005).

Heavy metals such as lead and mercury are known to perturb haem biosynthetic pathways and can serve as initiating factors for porphyria attacks. In 2005, researchers from several universities including the University of Kent published a study in which the metals content of King George's hair was analyzed. Though mercury levels were normal, and the level of lead was only slightly higher than would be expected, the levels of arsenic contained in the hair far exceeded the expected range. The arsenic content of King George's hair is consistent with arsenic poisoning (Cox et al., 2005), however the current hypothesis for the abundance of arsenic, as well as the precipitation of the king's porphyric attacks is far more interesting, and of much more importance to the understanding of how arsenic cycles in the environment.

Arsenic is the twentieth most abundant element in the earth's crust (Francesconi & Kuehnelt, 2002), with a natural abundance of 1.8 parts per million and increasing concentrations in areas of high volcanic activity (Goessler & Kuehnelt, 2002). Throughout history, arsenic has been associated with the extremes of industry, medicine and murder. Co-occuring with minerals of copper, Bronze Age smiths using primitive smelting operations would have, by chance, created copper-arsenic alloys. While the scientific knowledge of why a particular mineral gave a better alloy, the smiths would have been able to select for minerals that would have yielded alloys with the desirable arsenic to copper ratio. In ancient Egypt as well as China, arsenic was used to produce silver surfaces on mirrors and statues. Arsenic has seen use in the leather industry, painting, and arsenic sulfide (As_2S_3) as well as realgar (As_2S_2) were used by alchemists in hopes of extracting gold (Nriagu, 2002).

Arsenic has also been of historical importance as a medicinal agent. Initially used to treat external diseases of horse and sheep, arsenic eventually moved into a prominent role as a medicinal agent (Nriagu, 2002). Indeed, according to Nriagu (2002), when the nineteenth century closed every major disease known was subject to arsenotherapy; from external treatment of skin diseases to ingestion of Fowler's solution to treat anemia and leukemia.

However, arsenic's most well know association is with that of murder. From a prominent role in John Kesselring's play Arsenic and Old Lace to its fame as “inheritance powder,” the mere mention of arsenic carries with it images of murder and suicide. Until 1836, when James Marsh published a method for detecting low levels of arsenic (Taylor, 1875) arsenic remained the poison of choice for the upper class. While exact figures are

unknown, J Beckman (1846) estimates that 600 people were murdered by Aqua della Toffana, a generic name for poisons in Europe during the century between 1630 and 1730 (Nriagu, 2002).

Naturally occurring arsenic occurs in four oxidation states: As (-III), As(0), As(III) and As(V) (Oremland & Stolz, 2003), the most abundant of which is arsenate [As(V)] (Francesconi & Kuehnelt, 2002). Even though As(V) is the most commonly found form of natural arsenic, arsenite [As(III)] is found in relatively high abundance in anoxic sediments where microbiological processes reduce As(V) to As(III) (Oremland & Stolz, 2003). This shift demonstrates the microbiological cycling of the element from the pentavalent form to the trivalent form.

Arsenic in any form is toxic to most organisms, though some organisms possess resistance mechanisms to the toxin. Commonly, this system functions through some form of methylation followed by selective transport out of the cell (Newman, 2006) (Ratnaike, 2003). Interestingly, there are some organisms that can couple reduction of arsenate to arsenite with production of adenosine triphosphate (ATP), thus generating energy for the cell. At present, there are 24 microorganisms able to use arsenic as the terminal electron acceptor for production of ATP (Stolz et al., 2006). These organisms use arsenate much like oxygen is used by aerobic organisms; as the terminal electron acceptor in the aerobic electron transport chain (Oremland & Stolz, 2003). It is possible that these organisms mobilize mineralized arsenic into water supplies; a great concern in areas of West Bengal and Bangladesh, India. Within the United States, there is also concern of groundwater contamination.

Chakraborti and colleagues (2002) report that currently nine districts in West Bengal are impacted by arsenic contamination of their water sources. In 19 blocks of the region known as Murshidabad, 3.2 million people are served by an estimated 130,000 tube wells. Chakraborti and colleagues surveyed 22,274 of these wells, and found that a stagger sixty-six percent contained greater than ten parts per million. Of these wells, thirty-two percent had a concentration greater than fifty parts per million arsenic. The human health considerations of this are staggering (Chakraborti et al., 2002).

The researchers estimate that more than six million individuals are consuming water severely contaminated with arsenic, and that more than 300,000 individuals suffer from arsenical skin lesions. Of the 8,500 people clinically examined, 3,100 had identifiable skin lesions. In a single block in Murshidabad, 45 patients were likely suffering from Bowen's carcinoma, 13 suffering from non-healing ulcer and eight had already lost limbs to cancer or gangrene. In a single family of eighteen, all nine adults examined had sever arsenical skin lesions and were suspected to have Bowen's carcinoma (Chakraborti et al., 2002).

Arsenic contamination of groundwater in Bangladesh tells a similar story. Of 663 water samples analyzed, close to eighty percent had arsenic concentrations in excess of fifty parts per million. Again, extensive clinical examination of those affected revealed a high portion of the population suffering from arsenical skin lesions, ulcers and various forms of cancer (Chakraborti et al., 2002).

As discussed, the problems of high level arsenic exposure, exposure in excess of fifty parts per million, have been well documented in areas such as Bangladesh and West Bengal, India, arsenic at any levels are of concern. Recently, the Environmental

Protection Agency has enacted changes regarding permissible levels of arsenic in drinking water. These recent changes have suggested a non-enforceable target level of 0 parts per billion, with an enforceable level of ten parts per billion. It is important to note that these changes stem from rising health concerns regarding chronic exposure to low to moderate levels of arsenic, levels between ten and fifty parts per billion (EPA, 2001). These levels are of one magnitude less than those documented by Chakraborti.

In New England, concentrations of arsenic within this level are known to occur within groundwater. A study conducted to characterize regional occurrence of arsenic in aquifers in parts of New England found that of the 88 aquifers studied, 34% had arsenic concentrations in excess of 10 ppb, with the highest percentage exceeding this concentration being found in bedrock wells composed mainly of metamorphosed marine sediments (Ayotte et al., 2003). Arsenic levels in this range are implicated in a wide range of skin, bladder and lung cancers (Ratnaike, 2003).

What, then, is the connection of the arsenic redox cycle with a monarch that passed away over 300 years ago? The answer is in the mineralization of arsenic. Though arsenic is a relatively abundant element, it most frequently occurs in mineral form. Without the aid of microbial processes, mineralized arsenic is generally found in close association with several ores such as gold, copper, lead (Oremland & Stolz, 2003) or antimony (Cox et al., 2005). A close examination of King George III's medical records reveal he often took antimony to help with stomach pains (Cox et al., 2005).

Interestingly, previous estimates have indicated that the level of arsenic contamination in antimonial drugs may have reached as high as five percent during the period in which the king lived. Cox et al (2005) estimate this could lead to ingestion of

nearly nine milligrams of arsenic per day, and would thus lead to chronic high level arsenic exposure. While there has been no direct link between arsenic and onset of porphyric attacks, arsenic is known to react with sulphydryl groups in the haem biosynthesis pathway (Apostoli et al., 2002) (Woods & Southern, 1989). The current hypothesis is that the antimony he took was unable to be purified away from the closely associated arsenic based on chemical purification methods in place at that time. The metalloid arsenic then acted in much the same way as heavy-metals do and exacerbated his porphyrria (Cox et al., 2005).

Human Health Concerns

Arsenic is an element of great renown for its historical role as a poison. The potent poison arsenic trioxide has been referred to as "The Poison of Kings" and received acclaim as inheritance powder during the middle ages for its role in eliminating spouses, lovers and the heads of the great households (Nriagu, 2002). The minimum lethal amount is between sixty and eighty milligrams in a single dose (Ratnaike, 2003). However, because of its extended residence time, chronic exposure to lower levels, levels below fifty micrograms still leads to sever consequences. As previously mentioned, this is of very real concern both abroad in places such as West Bengal, India, but is also of much concern closer, in areas such as the New England region of the United States.

While the problems of high level arsenic exposure arsenic at any levels are of concern. As previously discussed, the recent EPA changes have suggested a non-enforceable target level of zero parts per billion arsenic in drinking water, with an enforceable level of ten parts per billion stemming from health concerns regarding

chronic low and moderate exposure (EPA, 2001). In many areas of the United States, drinking wells do not meet this criteria (Figure 2).

To understand the human health impact of arsenic, it is important to understand how the two prevalent arsenic oxidation states, arsenate [As(V)] and arsenite [As (III)], act at the cellular level. Initially absorbed in the small intestine by a protein gradient requiring electrogenic process, arsenic is generally biomethylated in the liver to monomethylarsonic acid (MMA) or dimethylarsonic (DMA) acid. These two compounds, while not completely harmless, are substantially less toxic than either arsenate or arsenite. However, it takes between three and five days for half of the arsenic ingested to be excreted in the urine. Because of this resonance time, low level exposure to arsenic can lead to chronically elevated levels in tissue (Ratnaike, 2003).

Chemically, arsenate is analogous to phosphorous. Consequently, arsenate is able to be substituted for phosphorous in compounds central to cellular metabolism (Ratnaike, 2003). Indeed, arsenate's toxicity is based in its ability to uncouple oxidative phosphorylation associated with the enzyme glyceradehyde 3-phosphate dehydrogenase subsequently disrupting a key step in glycolysis. Many bacteria have a detoxifying arsenate reductase mechanism, the arsC system, which reduces internal pools of arsenate to arsenite. Arsenite is then removed by an active ion pump (Oremland et al., 2002). Though arsenate's toxicity is based in its ability to mimic phosphorous and uncouple a key step of the glycolytic pathway, the more oxidized form of arsenic, arsenite, binds thiol groups within proteins (Oremland & Stolz, 2003). Consequently, many bacteria have developed novel modes of detoxification. Because arsenate is less toxic than

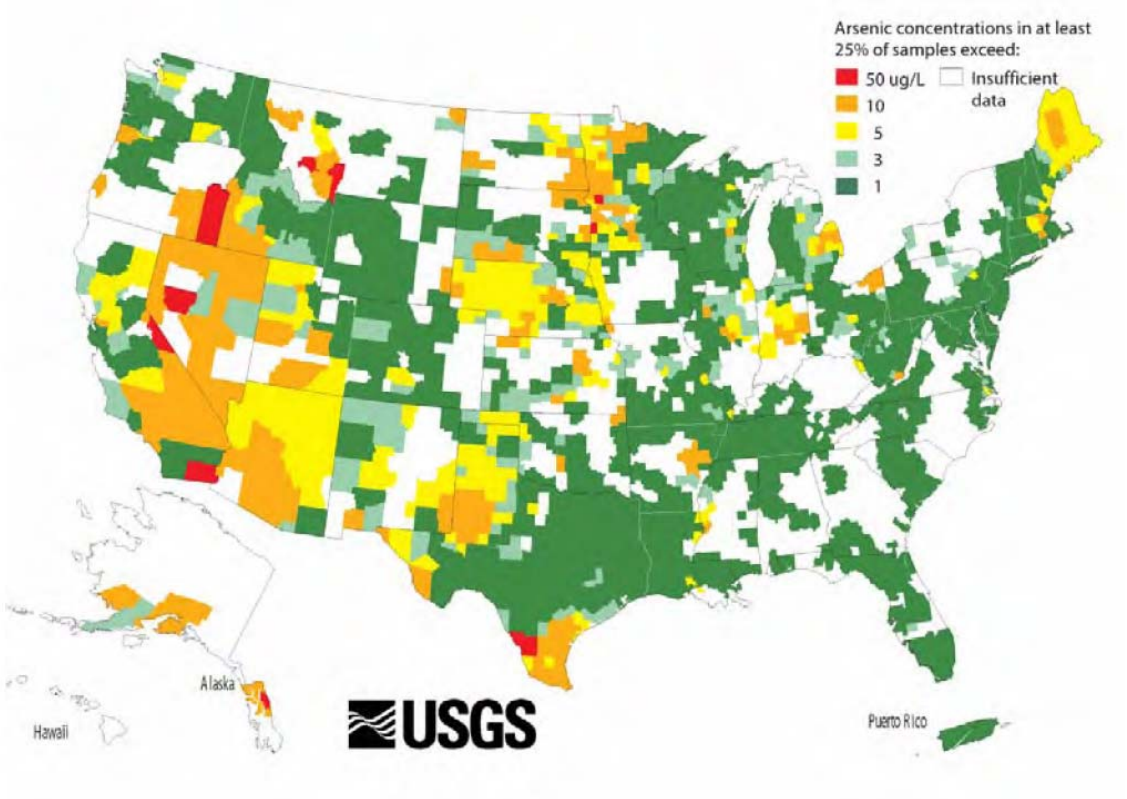


Figure 2: Arsenic concentrations in the United States. (Chou et al., 2005).

arsenite, some organisms are able to oxidize arsenite to arsenate, using oxygen as the terminal electron acceptor. Of more importance, especially in organisms that reduce arsenate to arsenite as a detoxification mechanism, is the function of ArsB.

The most thoroughly studied *ars* operon is that of plasmid R773 which consists of five genes: *arsR, arsD, arsA, arsB* and *arsC*. While not all operons in living systems contain all five genes, representative sequences suggest that, at a minimum ArsC, must be expressed for arsenate resistance. Ars C is a small cytoplasmic arsenate reductase that catalizes the reaction from As(V) to As(III). Indeed, it is the only protein of the *ars* operon to catalyze such a reaction. By reducing As(V) to As(III), the As (III) is then selectively exported out of the cell by either ArsB alone, or with the heteromultimer ArsAB pump (Silver et al., 2002).

ArsB is a membrane protein involved in arsenite and antimonite resistance. By pumping these metalloid oxyanions from the cells (Silver et al., 2002) the cell is able to lessen the internal pool of arsenite in the cytosol. ArsB alone selectively passes arsenite out of the cell, deriving energy solely from the cell's membrane potential. Interestingly, in the presence of a functioning *arsA* gene, an ArsAB complex forms. Deriving energy from hydrolysis of adenosine tri-phosphate (ATP), this transport protein is able to actively pump arsenite out of the cell without relying on a pre-established gradient for the energy requirement (Silver et al., 2002).

Less studied in this system are the roles of ArsR and ArsD. The evidence would suggest that these proteins may act in regulation of the operon. Indeed, Silver reports that As(III) binding to ArsR induces a conformational change. This conformational shift results in release of the repressor protein, ArsD, from the operator DNA. As yet, no

evolutionary importance has been assigned to ArsD as its absence has little effect underlaboratory conditions. However, the high frequency of this protein suggests a subtle role in selection that is not yet understood (Silver et al., 2002).

A survey of the literature reveals the importance that this theme of detoxification and removal plays an important role in bacterial resistance mechanisms to both arsenite and arsenate. Additionally, it is interesting to note that eukaryotes possess several resistance mechanisms which are not homologous to the mechanisms seen in prokaryotes, but have evolved independently. In Saccharomyces cerevisiae, this theme continues whereby Arr2 (previously Acr2p) reduces arsenate to arsenite, and the ARR3 (previously ACR3) gene product catalyzes arsenite extrustion from the cell (Silver et al., 2002).

Given that arsenic is a known carcinogen it is readily understood why there is such diversity and importance put on the role of detoxification and removal at the cellular level. It is interesting, though, to realize that neither arsenate nor arsenite directly damage deoxyribonucleic acid (DNA). However, exposure to arsenic leads to the inactivation of up to two hundred enzymes. Of these, the most noted are thos that are involved in deoxyribonucleic acid replication and repair (Ratnaike, 2003). Recent evidence has also suggested that arsenic may play a role in altering the function of enzymes. One such example is altered function of the glucocorticoid receptor (GR) in response to arsenite exposure.

To examine the effect of arsenic on the glucocorticoid receptor, Kaltreider and colleagues created luciferase constructs that were under control of a glucocorticoid response element. Using the synthetic glucocortoid dexamethasone, the researchers were able to monitor the effect of dexamethasone on cells exposed to dexamethasone alone, or

pre-treated with non-cytotoxic doses of arsenite, levels below 3.3 μM. The researchers demonstrated that pre-treatment with varying concentrations of an arsenite pre-treatment yielded correspondingly lower expression of the GRE:luciferase construct despite subsequent studies demonstrating that glucocorticoid concentration and translocation remained unaffected. These results suggest that arsenite may interfere with the receptors ability to initiate nuclear transcription, though the mechanism behind this remains unresolved (Kaltreider et al., 2001).

In large part, the actual mechanisms behind arsenic toxicity remain unknown. As previously discussed, arsenic does not directly damage DNA, but does inactivate enzymes as well as alter the function of other enzymes. Nonetheless, acute and chronic arsenic exposure results in various clinical manifestations. Generally, acute poisoning of less than five milligrams results in vomiting and diarrhea. However, these symptoms resolve in less than twelve hours, and no treatment is necessary. At the lethal level of acute poisoning, levels greater than one hundred milligrams, patients invariably present with problems of the gastrointestinal system including vomiting and diarrhea. Ultimately, death results from severe dehydration at these levels of exposure (Ratnaike, 2003).

Of greater importance is the clinical manifestation of those exposed to chronic low-level arsenic. In patients suffering from chronic low-level arsenic poisoning, affects to most major systems of the body are reported. Initially, as with acute poisoning, patients typically present with gastrointestinal problems including diarrhea and vomiting. Left untreated, however, arsenic leads to changes in the cardiovascular system, neurological system, genitourinary system, respiratory system and the endocrine systems.

Compounding this, in regions where chronic low-level exposure is prevalent, fetuses, infants and children are exposed to increasingly higher levels of arsenic from their mother. In the Andes, another locale in which chronic exposure is prevalent, mothers drinking water containing arsenic concentrations of approximately two hundred parts per billion had elevated levels of placental arsenic. Additionally, the toxin is passed to infants and children through breast milk further exposing the children to low levels of arsenic (Ratnaike, 2003).

Industrial Uses of Arsenic

Historically, arsenicals have seen use as pesticides starting in the mid nineteenth century and continuing until the advent of organic pesticides cause the use of them to wan in the 1940's. Among these, calcium arsenate, lead arsenate, sodium arsenite and sodium arsenate were the dominant compounds used. Owing largely to an increased awareness of human health and environmental risks associated with continued use of these compounds, use has largely disappeared since 1960 (Chou et al., 2005). In 1993, amid growing concern, food uses and use on cotton plants were voluntarily halted (Chou et al., 2005).

However, despite arsenic's toxicity it continues to see a wide variety of industrial applications. The United States alone used 21,600 metric tons in 2003. By far the leading use, laying claim to over 90% of arsenic application in 2003, was for production of chromated copper arsenate (CCA), a wood preservative. The remaining 10% resulted in the production of agricultural products such as fertilizers, herbicides and insecticides. According to the 2005 draft toxicity report, CCA is widely used as a wood preservative

throughout the world. In 1997, 20.6 million cubic meters of wood were treated with CCA, and marketed as pressure treated (Chou et al., 2005). Though CCA manufacturers began a volunteer transition from CCA to other preservatives in 2003, there has been a lag in such actions impacting other industries. Among these uses is the use of the organoarsenical centrally important to this study, roxarsone, as an antimicrobial feed additive in the poultry industry.

While roxarsone was initially approved for use in the mid-1960s as an antimicrobial, it has recently come under much investigation. Anderson and Chamblee (2001) conducted a study involving arsenic deposition patterns in chicks fed roxarsone versus those not fed roxarsone. Of the possible deposition patters, the pattern observed was consistent with what the researchers expected for organisms with no tissue absorption of the metalloid. As the chicks grew and had higher food consumption, more arsenic was deposited in their feces. However, after being given the withdrawal food, food that contained no roxarsone, total arsenic concentration started to drop. However, it is interesting to note that the samples taken prior to slaughter contained approximately 6 parts per million (ppm) of arsenic, while samples taken from the control groups had an experimental high of 0.53 ppm (Anderson & Chamblee, 2001).

Current Research

As previously mentioned, roxarsone is an organoarsenical that has seen widespread use in the poultry industry; initially for the prevention of coccidiosis infections, but later to stimulate increased broiler growth and plumage coloration

(Salsbury Laboratories, 1981). The dosage of roxarsone ranges from 22.7 to 45.4 g of roxarsone per ton of chicken feed (Alpharma, 1999). According to Garbarino et al (2003) estimates, this equates to approximately 9 x 105 kg of Roxarsone, or 2.5 x 105 kg of arsenic on the soils of the United States from the manure (Garbarino et al., 2003). Hardest hit with this arsenic deposition are those states which lead the nation in poultry production (Figure 3).

In 1969, Morrison concluded that use of roxarsone did not increase the arsenic levels in soil or crops. Fields having poultry litter (15 to 30 mg/kg arsenic) applied at a rate of 4 to 6 tons per year for twenty years were compared to fields having no history of poultry litter treatment. While the soil, alfalfa crop and clover crop showed no significant differences in arsenic concentrations between the fields, drainage from the treated fields contained 0.29 mg/L of arsenic (Morrison, 1969). Similarly, in a study of roxarsone transport in agricultural watersheds, Brown et al (2005) found that there was not a significant difference in total arsenic concentration in soils treated with poultry litter compared to those not treated with poultry litter (Brown et al., 2005). However, another study suggested that there is an increase in soil arsenic levels in fields treated with poultry litter (Gupta & Charles, 1999).

Recent evidence from our laboratory, and others, has suggested that microbial transformation of the organoarsenical can occur under anaerobic conditions (Figure 4). Garbarino et al (2003) demonstrated that soils treated with poultry litter transformed 90% of roxarsone present in composting experiments into As(V) in 23 days. Complete transformation occurred by day 38 (Garbarino et al., 2003). Studies have also identified several intermediates either existing in the degradation pathway, or as an alternate

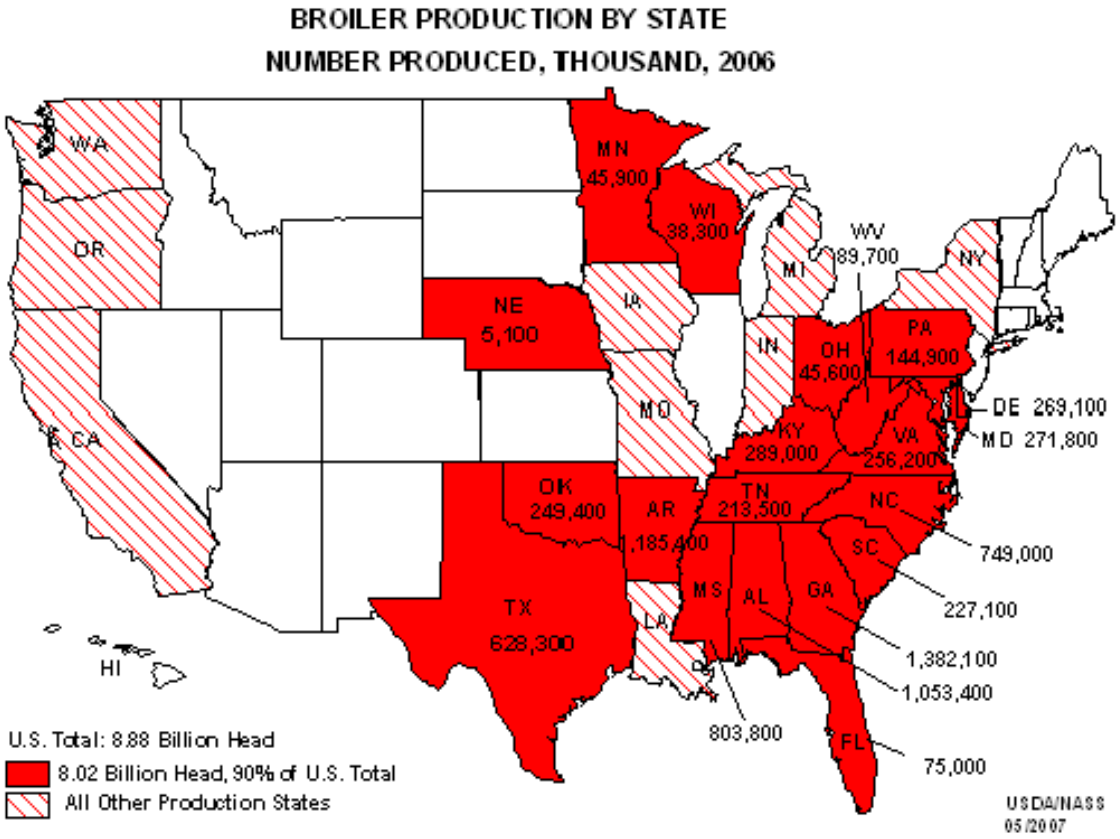


Figure 3: Map of United States Poultry Production.

http://www.nass.usda.gov/Charts_and_Maps/Poultry/brlmap.asp

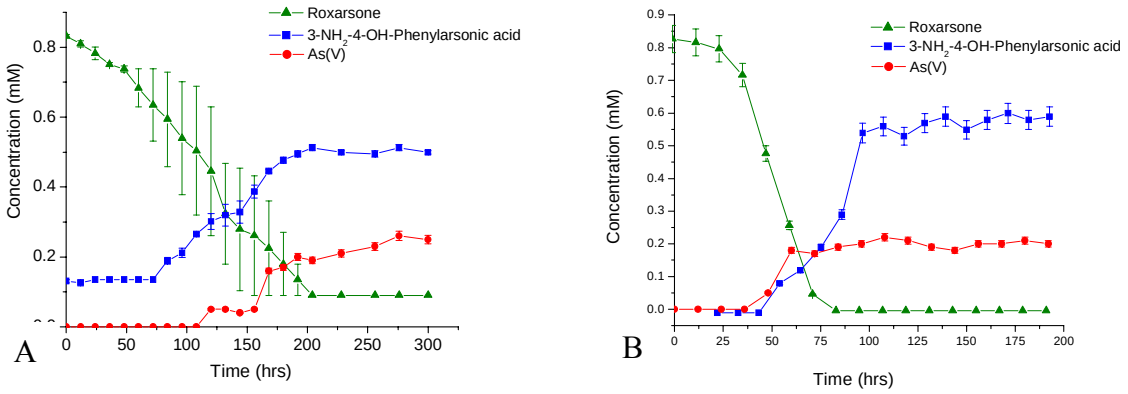


Figure 4: Anaerobic transformation of roxarsone. Chicken litter enrichments (A) and cultures of *Clostridium* sp. OhILAs (B) (Stolz et al 2007). Error bars represent standard error of the mean (SEM) as calculated from triplicate cultures.

terminal transformation product (Rosal et al., 2005) (Stolz et al., 2007). Some of these compounds, such as 3-A-4-HBAA, are readily soluble in water (Rutherford et al., 2003) further contributing to increased arsenic levels in waterways and raising health concerns, both human and environmental, around the United States.

Cortinas and colleagues (2006) found that enrichment cultures obtained from sewage sludge were capable of transforming roxarsone. Cultures grown under methanogenic, methanogenic plus lactate, or sulfate reducing conditions demonstrated almost complete transformation of roxarsone as determined by high performance liquid chromatography (HPLC) in less than twenty-five days. Abiotic, heat-killed and cultures grown under denitrifying conditions showed little to no transformation.

HPLC of the resulting media demonstrated that on day ten, the cultures had produced between 0.5 and 0.6 mM of 3A4HBAA from the initial concentration of roxarsone (1 mM), with continued production of 3A4HBAA to the final data point on day sixteen. Ultimately, the molar yield of 3A4HBAA was equal to 86% of the roxarsone removed from the cultures. Subsequently, the researchers investigated the long-term stability of the transformation compounds and found extensive transformation of 3A4HBAA in extended incubation cultures to levels below 0.2 mM (Cortinas et al., 2006). Unfortunately, the microbial composition of the enrichments was not identified. While the chicken cecum has a vastly diverse microbial community, it has been well established that *Clostridia* are the dominant species of the chicken cecum. Here, we have examined the microbial transformation of roxarsone using a pure strain of *Clostridium*, *Clostridium* sp. strain OhILAs .

Preliminary data from our laboratory suggests that in anaerobic cultures inoculated with OhILAs, as well as cultures inoculated from a chicken litter enrichment slurry (received from M. Schreiber at Virginia Polytechnic Institute and State University) transform roxarsone at pH 7.3. Chicken litter cultures have shown rapid transformation of 1 mM roxarsone into 3-A-4-HBAA and As(V) (Figure 4A). Interestingly, As(V) began at 84 hours was preceeded by production of 3-A-4-HBAA appearing at 72 hours. By 192 hours, phenol was detected (Figure 4A). A similar pattern was noticed for the cultures inoculated with OhILAs (Figure 4B). Additionally, As(III) was detected after 48 hours (Figure 4B). No abiotic transformation was detected in heat-killed cultures.

While great progress has been made in this field of arsenic respiring microbes, there is still quite a bit of work that needs to be done. Currently, several microbes have been identified that will transform arsenate [As(V)] into the more toxic arsenite [As(III)]. Similarly, it has been identified that certain strains of *Clostridium* sp., as well as microorganisms in chicken litter, have the ability to transform roxarsone into a variety of end products, including 3-A-4-HBAA (3-A-4-HBAA). However, the specific mechanism behind this has yet to be determined, though a likely mechanism has been proposed. In this model, the nitro group on the aromatic ring is first reduced to an amine to form 3-A-4-HBAA (Figure 5). Though evidence from previous studies suggests this is an acceptable first step, the remainder of the pathway, that of deamination of the aromatic ring and subsequent release of the arsenic group, is entirely speculative. While this will take many more years of research to reach an acceptable model, the current study provides critical framework from which to build such a model.

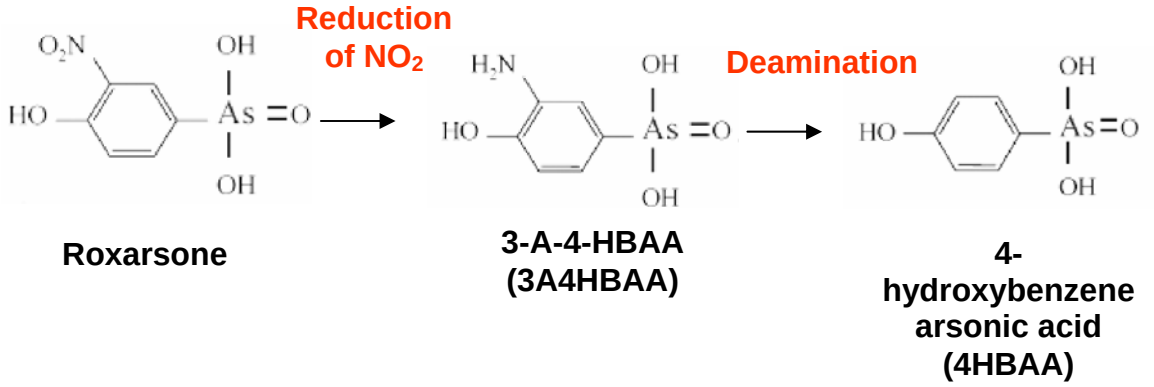


Figure 5: Proposed mechanism of roxarsone transformation. Reduction of the nitro group is followed by deamination of 3-A-4-HBAA. Subsequent release of the arsenic group may occur.

Master's Thesis Specific Aims

1. Determine if other carbon substrates (e.g., glycerol, fructose, pyruvate, butyrate) can serve as electron donors to the reduction of roxarsone.

2. Determine whether growth supplements (e.g., selenium) are necessary for *Clostridium* sp. OhILAs to transform 3-amino-4-hydroxyphenylarsonic acid or 4-hydroxyphenylarsonic acid.

3. Determine whether the respiratory arsenate reductase and resistance arsenate reductase appear in the proteome of *Clostridium* sp. OhILAs when grown in the presence of organoarsenicals (e.g., roxarsone, 3-amino-4-hydroxyphenylarsonic acid, 4-hydroxyphenylarsonic acid)

Materials and Methods

Cell Culture Experiments:

For all cell culture experiments, the organisms were grown at ambient temperatures on either a minimal media (OhBs) containing or a complex (OhSt) media. In one liter, OhBs contained 0.095 g magnesium chloride (Fisher Scientific, Hanover Park, IL; FW 203.31), 4.2 g sodium bicarbonate (Fisher Scientific; FW – 84.01), 1 g yeast extract (Sigma-Aldrich, St. Louis, MO) and 10 ml of a 100X mineral mix (appendix 1) and 2 ml of a 500X vitamin mix (appendix 1). OhSt contained in one liter 0.225 g potassium phosphate monobasic (Sigma Aldrich; FW 136.1), 0.225 g potassium phosphate dibasic (Sigma Aldrich; FW 136.1), 1 g sodium chloride (Fisher Scientific catalog; FW 58.44), 0.178 g ammonium chloride (Fisher Scientific; FW 53.49), 0.095 g magnesium chloride, 3 g yeast extract, 4.2 g sodium bicarbonate, 0.2 g sodium sulfide (Sigma Aldrich) and 1 ml of a 1 mM sodium selenite (Sigma Aldrich; FW 172.94), 10 ml of a 100X mineral mix (appendix 1) and 2 ml of a 500X vitamin mix (appendix 1). The pH of the prepared media was adjusted to 7.5 and 100 ml aliquots were degassed with high purity nitrogen (Airgas, Radnor, PA) passed through heated copper coils to remove any contaminating oxygen. Media was degassed for ten minutes in 125 ml crimp-top sealable bottles. After the bottles were sealed, the media was then heat sterilized at 121^{o}C and 20 psi for forty-five minutes. After cooling to ambient temperatures, necessary amendments were made using stock solutions prepared in either OhSt or OhBs transferred using a Beckton-Dickinson 3 ml syringe and a FisherBrand 0.22um syringe filter (pre-sterilized). All cell culture experiments were conducted in triplicate with a

sterile (uninoculated) sample as control. Cultures were swirled by hand prior to sampling to ensure equal distribution of the organisms. Figure 6 illustrates the experimental design for the cell culture experiments.

Chicken Litter Enrichment ~ roxarsone

Here, the ability of microbial enrichments obtained from chicken litter to transform roxarsone was investigated using lactate as the initial carbon source. Chicken litter from the Shenandoah Valley of Virginia, was obtained from Madeline Schreiber, Associate Professor of Hydrogeosciences, Virginia Polytechnic Institute and State University, Blacksburg, Virginia. A 100 ml bottle of OhBs media was temporarily unsealed, and five grams of chicken litter was added to form a chicken litter slurry. The bottle was immediately resealed, and allowed to incubate at ambient temperature for thirty minutes to one hour before being used to inoculate experimental cultures. This time allowed aerobic organisms in the culture to deplete any introduced oxygen and re-established an anoxic atmosphere.

100 ml aliquots of OhBs media were amended as previously described with 1 ml of a 1M stock solution of sodium lactate (Sigma Aldrich), prepared in OhBs, to a final concentration of 10 mM. roxarsone (Acros Organics, Morris Plains, NJ; FW 263.03) containing cultures were also amended as previously described with 2 ml of a 50 mM stock solution of roxarsone, to a final concentration of 1 mM. After amendment, experimental cultures were inoculated with 1 ml of the chicken litter slurry transferred aseptically via a 3 ml Beckton-Dickinson syringe.

Experimental Method:

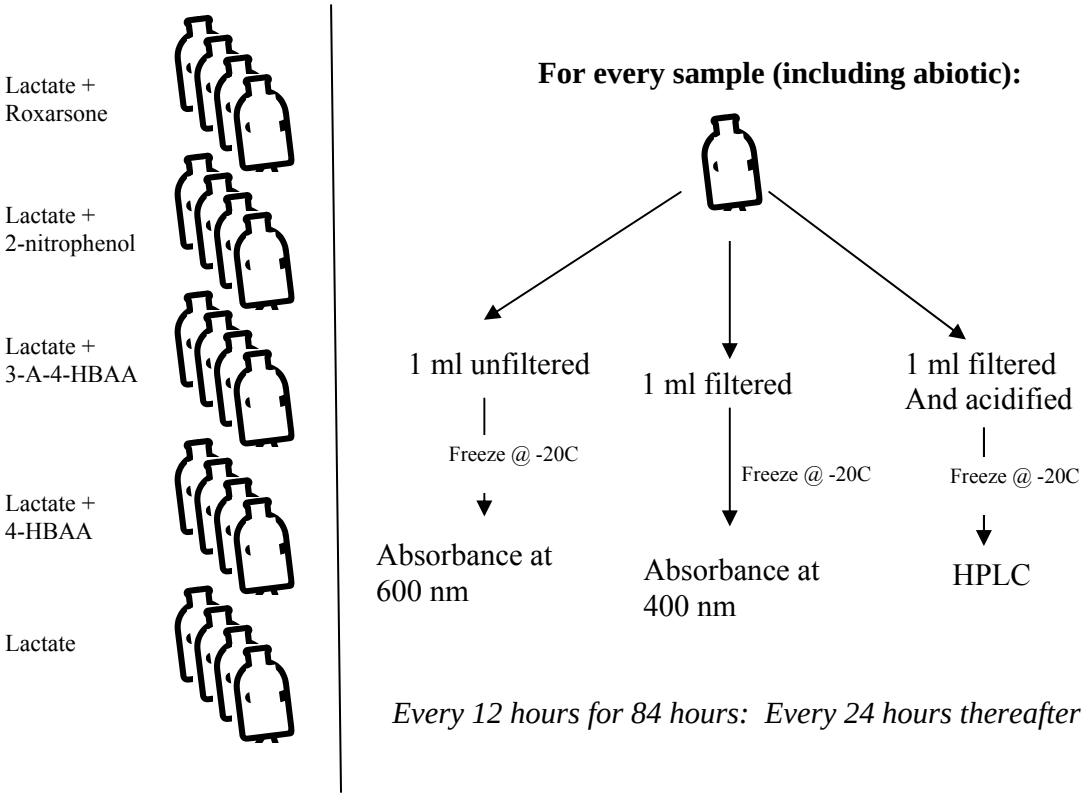


Figure 6: Cell culture experimental design. Triplicate experimental cultures were incubated at ambient temperatures. 3 ml of sample collected at specified time points for duration of experiment. 1 ml reserved for turbidimetry, 1 ml filter sterilized using a 0.22 μm syring filter and 1 ml was filter sterilized and acidified with 200 μl of 2 N sulfuric acid.

Samples were collected initially and at specified timepoints throughout the duration of the experiment by withdrawing 3 ml of the experimental and abiotic control cultures using a syringe. A 1 ml aliquot of the sample was immediately transferred to a FisherBrand microcentrifuge tube and stored at -20^{o}C until spectrophotometric analysis. Another 1 ml aliquote was filter sterilized using a FisherBrand 0.22 μm syringe filter and stored at -20^{o}C until spectrophotometric analysis. An additional 1 ml aliquot was needed for high performance liquid chromatography analysis. This aliquot was filter sterilized and acidified using 200 μl of a 2N sulfuric acid (H_2SO_4) (Fisher Scientific) solution. These samples were stored in glass vials at -20^{o}C.

Clostridium sp. strain OhILAs pure culture experiments ~ roxarsone

Here, the ability of pure strain *Clostridium* sp. strain OhILAs to transform roxarsone was investigated using lactate or fructose (Fisher Scientific; FW 180.16) as the initial carbon source and electron donor where indicated. Stock cultures were maintained on OhSt prepared under an atmosphere of nitrogen. Prior to transfer, purity of strain was determined using phase contrast microscopy. Slides were prepared using 1 drop of the stock culture and observed at a total magnification of 400X.

100 ml aliquots of OhBs media were amended as previously described with 1 ml of a 1M stock solution of sodium lactate or fructose as indicated, prepared in OhBs, to a final concentration of 10 mM. roxarsone containing cultures were also amended as previously described with 2 ml of a 50 mM stock solution of roxarsone, to a final concentration of 1 mM. After amendment, experimental cultures were inoculated with 1

ml of the stock culture at log phase was transferred aseptically via a 3 ml Beckton-Dickinson syringe.

Samples were collected initially and at specified timepoints throughout the duration of the experiment by withdrawing 3 ml of the experimental and abiotic control cultures using a syringe. A 1 ml aliquot of the sample was immediately transferred to a FisherBrand microcentrifuge tube and stored at -20^{o}C until spectrophotometric analysis. Another 1 ml aliquot was filter sterilized using a FisherBrand 0.22 μm syringe filter and stored at -20^{o}C until spectrophotometric analysis. An additional 1 ml aliquot was needed for high performance liquid chromatography analysis. This aliquot was filter sterilized and acidified using 200 μl of a 2N sulfuric acid (H_2SO_4) solution. These samples were stored in glass vials at -20^{o}C.

Cell Turbidity Measurements

Turbidity was monitored at 600 nm using a Perkin Elmer Lambda 2S dual band spectrophotometer and standard 1.5 ml quartz cuvettes with a 1 cm pathlength. The spectrophotometer was zeroed using 1 ml of sterile OhBs as reference. Unfiltered samples from pure culture experiments were thawed and stored on ice except during analysis. Absorbance of the samples at 600 nm was recorded. The data was graphed using GraphPad Prism 5.0 using the "Three Replicates" method to calculate standard error.

Microbial transformation of 2-nitrophenol

2-nitrophenol (Acros Organics catalog; FW 139.11) is a nitroaromatic that is similar in structure to roxarsone with the notable exception that this compound lacks an arsenic group. Here, the ability of microbial enrichments obtained from chicken litter and pure strain *Clostridium* sp. strain OhILAs to transform additional organoarsenicals present in the proposed degradation pathway was investigated using lactate or fructose as the initial carbon source as indicated. Chicken litter slurry and stock cultures of OhILAs were prepared as previously described.

100 ml aliquots of OhBs media were amended as previously described with 1 ml of a 1M stock solution of sodium lactate or fructose as indicated, prepared in OhBs, to a final concentration of 10 mM. 2-nitrophenol containing cultures were also amended as previously described with 2 ml of a 50 mM stock solution of 2-nitrophenol, to a final concentration of 1 mM. After amendment, experimental cultures were inoculated with 1 ml of the chicken litter slurry or stock culture of log phase *Clostridium* sp. strain OhILAs was transferred aseptically via a 3 ml Beckton-Dickinson syringe. Samples were collected as previously described.

Microbial transformation of 3-A-4-HBAA and 4-hydroxybenzene arsonic acid

Here, the ability of microbial enrichments obtained from chicken litter and pure strain *Clostridium* sp. strain OhILAs to transform additional organoarsenicals present in the proposed degradation pathway was investigated using lactate or fructose as the initial carbon source as indicated. Chicken litter slurry and stock cultures of OhILAs were prepared as previously described.

90 ml aliquots of OhBs media were amended as previously described with 1 ml of a 1M stock solution of sodium lactate or fructose as indicated, prepared in OhBs, to a final concentration of 10 mM. 3-A-4-HBAA (City Chemical, LLC) containing cultures were also amended with 10 ml of a 10 mM stock solution of 3-A-4-HBAA to a final concentration of 1 mM.

100 ml aliquots of OhBs media were amended as previously described with 1 ml of a 1M stock solution of sodium lactate or fructose as indicated, prepared in OhBs, to a final concentration of 10 mM. 4-hydroxybenzene arsonic acid (City Chemical, LLC) containing cultures were also amended with 1 ml of a 100 mM stock solution of 3-A-4-HBAA to a final concentration of 1 mM.

After inoculation with 1 ml of chicken litter slurry or 1 ml of log phase OhILAs stock culture, samples were collected as previously described.

Aerobic Chicken Litter Enrichment ~ roxarsone

Here, the ability of microbial enrichments obtained from chicken litter to transform roxarsone under aerobic conditions was investigated using lactate as the initial carbon source. Aerobic OhBs media was prepared as previously described without degassing. Aerobic chicken litter slurry was prepared as previously described, without degassing.

100 ml aliquots of OhBs media were amended as previously described with 1 ml of a 1M stock solution of sodium lactate, prepared in OhBs, to a final concentration of 10 mM. roxarsone containing cultures were also amended as previously described with 2 ml of a 50 mM stock solution of roxarsone, to a final concentration of 1 mM. After

amendment, experimental cultures were inoculated with 1 ml of the chicken litter slurry transferred aseptically via a 3 ml Beckton-Dickinson syringe.

Samples were collected initially and at specified timepoints throughout the duration of the experiment by withdrawing 3 ml of the experimental and abiotic control cultures using a syringe. A 1 ml aliquot of the sample was immediately transferred to a FisherBrand microcentrifuge tube and stored at -20^{o}C until spectrophotometric analysis. Another 1 ml aliquote was filter sterilized using a FisherBrand 0.22 μm syringe filter and stored at -20^{o}C until spectrophotometric analysis. An additional 1 ml aliquot was needed for high performance liquid chromatography analysis. This aliquot was filter sterilized and acidified using 200 μl of a 2N sulfuric acid (H_2SO_4) solution. These samples were stored in glass vials at -20^{o}C.

Colorimetric assay for determination of roxarsone concentration

Full wavelength scans of roxarsone using a Perkin Elmer Lambda 2S dual band spectrophotometer and standard quartz cuvettes with a 1 cm pathlength have shown that it has a peak absorbance at approximately 400 nm (Fisher, 2006). A standard curve of absorbance at 400 nm versus concentration was constructed using the following concentrations of roxarsone: 0.5 M, 0.25 M, 0.15 M, 0.1 M, 0.050 M and 0.025 M. The standard curve was constructed in GraphPad Prism using the "Single Values" method. A linear regression was run and and an extinction coefficient was calculated as the slope of the linear regression. Filtered samples from pure culture experiments were diluted one in ten and absorbance at 400 nm was recorded. Concentration of roxarsone was calculated using Beer's Law of

$$A / \epsilon l = concentration$$

where A is equal to the absorbance, l is equal to the pathlength of the cuvette (1 cm) and ε is equal to the previously determined extinction coefficient of the compound. To compensate for the dilution factor, this value was multiplied by ten.

Colorimetric assay for determination of 2-nitrophenol concentration

Full wavelength spectrophotometric scans of 2-nitrophenol have shown that it has a peak absorbance at approximately 417 nm. A standard curve of absorbance at 410 nm versus concentration was constructed using the following concentrations of 2-nitrophenol: 500 μM, 250 μM, 150 μM, 100 μM and 50 μM. The standard curve was constructed in GraphPad Prism using the "Single Values" method. A linear regression was run and and an extinction coefficient was calculated as the slope of the linear regression. Concentration of 2-nitrophenol was calculated using Beer's Law of

$$A / \epsilon l = concentration$$

where A is equal to the absorbance, l is equal to the pathlength of the cuvette (1 cm) and ε is equal to the previously determined extinction coefficient of the compound.

Investigations into the proteome of Clostridium sp. strain OhILAs

To investigate changes in the proteom of OhILAs grown under 10 mM lactate, 10 mM lactate and roxarsone, 10 mM lactate and 2-nitrophenol or 10 mM lactate and 5 mM sodium arsenate conditions, 1.8 L cultures of a complex lactate containing media (OhProt) comprised of OhSt media with the addition of 2.8 ml per liter 60% sodium lactate (Sigma Aldrich) prior to autoclaving was prepared. The media was placed in a 1.8

L crimp-top sealable anaerobic culture bottle and degassed with nitrogen for ten minutes using a diffuser. After degassing, the bottles were sealed and the media was heat sterilized at 121^{o}C and 20 psi for sixty-five minutes. The media was then amended with stock solutions prepared as described earlier to final concentrations of 10 mM lactate alone or 10 mM lactate and 1 mM of either roxarsone, 2-nitrophenol or sodium arsenate.

Cultures were incubated at ambient temperature for 72 hours. The cultures were then decanted into 1L centrifuge bottles and centrifuged at 7200 revolutions per minute (rpm) for thirty minutes at 4^{o}C. The supernatant was decanted and the cell pellet was re-suspended in approximately 15 ml of a buffer (lysis buffer #1) containing 20 mM Tris-HCl (Fisher Scientific; FW 121.14), 100 mM potassium chloride (J. T. Baker, Phillipsburg, NJ; FW 74.56), 0.1 mM ethylenediamine tetraacetic acid (EDTA) (Fisher Scientific; FW 372.24) and 1 mM dithiothreitol (DTT) (ACROS; FW 154.24).

After like cultures had been combined into 50 ml conical tubes, the cells were washed three times. To wash the cells, the cell suspension was centrifuged again at 7200 rpm for ten minutes at 4^{o}C. The buffer was decanted and replaced with fresh lysis buffer #1. After the final wash, the cell pellet was stored at -80^{o}C.

Cell lysis and fractionation

To separate the soluble and insoluble fractions, a combination of French Pressure Cell Lysis technique (French press) and ultracentrifugation was used. The pellets were allowed to thaw at 4^{o}C and resuspended in 3 ml of lysis buffer #1. The components of the mini-cell apparatus for French press were incubated in ice for 20 minutes prior to French pressing the cell suspension. For each sample, the cell suspension was placed into

the assembled mini-cell apparatus. Cells were lysed at an internal pressure of 15,000 psi (750 psi external) through two consecutive passes.

The lysed cell suspension was then placed into an ultracentrifuge tube. Tubes were balanced to within 0.005 g using additional lysis buffer #1 where necessary. Protein suspensions were spun at 40,000 rpm (150,00 x gravity) in a Beckman Class S ultracentrifuge using rotor 50.2Ti for 2 hours. The supernatant containing the soluble fraction of proteins was decanted and immediately stored at -80^{o}C for further experiments. The insoluble pellet was resupended in 1 ml of lysis buffer #1 and immediated stored at -80^{o}C for further experiments.

Quantification of proteins

In all cases, concentration of protein in both soluble and insoluble fractions were quantified using the BioRad Quick Start Protein Assay Kit. As per the kit's instructions, a standard of curve of 20 µl of 2, 1.5, 1, 0.75, 0.5, 0.25, 0.125 µg/µl concentrations was prepared using 20 µl of nanopure water as a blank. The necessary volume of included prepared dye was allowed to warm to ambient temperature. 1 ml of this dye was added to the 20 µl of either sterile water or protein standard and allowed to incubate at room temperature for at least 15 minutes but no longer than one hour. Standard quartz cuvettes of 1cm pathlength were used and the spec was zeroed using the 20 µl nanopure water as the reference cuvette. All standards were prepared in duplicate. A standard curve of the absorbance at 595 nm versus protein concentration was prepared using GraphPad Prism 5.0. The extinction coefficient (0.5935 L mole^{-1} cm^{-1}) was calculated as the slope of the linear regression.

Five microliters of unknown concentration of protein suspension was diluted to 20 µl with lysis buffer #1. A reference of 20 µl of lysis buffer #1 was prepared. To each of the unknown protein samples, and reference, 1 ml of the BioRad Quick Start Protein Assay Dye warmed to ambient temperatures was added. Samples were allowed to incubate at ambient temperature for at least 15 minutes but no longer than 1 hour. Using the 20 µl lysis buffer #1 sample as reference, the absorbance at 595 nm of the samples of unknown concentration was recorded. The unknown concentration was calculated using Beer's Law.

$$A/\epsilon l = concentration$$

This concentration was then multiplied by four to account for the one in four dilution compared to the standard curve.

Precipitation of proteins by trichloroacetic acid

To remove lipids and concentrate protein samples, samples were subjected to precipitation by trichloroacetic acid where noted. A volume of sample equal to 500 µg of protein was brought to a 1 ml volume with lysis buffer #1. To this, 50 µl of a 100% trichloroacetic acid (TCA) was added and samples were incubated on ice for the amount of time noted in subsequent experiments. The samples were spun at 7200 rpm at 4^{o}C for ten minutes and the supernatant was decanted. The pellet was washed with 100% acetone chilled to -20^{o}C and centrifuged again at 7200 rpm at 4^{o}C for ten additional minutes. The acetone was then decanted and the pellet was allowed to air dry at ambient temperature for approximately five minutes. The pellet was then resuspended in the experiment specific buffer as noted in subsequent experiments. Several samples were

prepared simultaneously. Protein yield and concentration were determined as previously described using protein suspended in a buffer (lysis buffer 2d) containing 7M urea (Fisher Scientific; FW 60.06), 2M thiourea (Fisher Scientific; FW 76.12), 4% (w/v) 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesul-fonate (CHAPS) (AppliChem, Cheshire, CT; FW 614.89) and 10 mM DTT.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis of proteins

To separate proteins in a polyacrylamide matrix, two primary methods of gel electrophoresis were employed, sodium dodecyl sulfate polyacrylamide gel electrophoreses (SDS-PAGE) and two-dimensional (2D) PAGE. For SDS-PAGE 7 cm gels, pre-cast gels were purchased from BioRad Laboratories (Hercules, CA). Precast 7.5% acrylamide ten well gels were purchased from BioRad laboratories as were 12% gels with ten wells and fifteen wells. All precast gels came with a 4% acrylamide stacking gel.

Protein fractions that did not undergo TCA precipitation were used for all native gels where indicated, while TCA precipitated suspensions were used for all denaturing gels where indicated. A volume of suspension equal to 70 µg (for Coommassie staining) or 5 µg (silver staining) was diluted in the appropriate buffer. For native SDS-PAGE, a buffer (native buffer) containing in one ml 250 µl of a 0.5 M Tris-HCl (pH 6.8) buffer, 300 µl of a 20% sodium dodecyl sulfate (SDS) (Sigma Aldrich; FW 288.38), 200 µl of 100% glycerol (Sigma Aldrich) and 250 µl of nano pure water. For denaturing SDS-PAGE, a buffer (denaturing buffer) containing in one ml 250 µl of a 0.5 M Tris-HCl (pH

6.8) buffer, 300 µl 20% SDS, 200 µl 100% glycerol, 100 µl 2-mercaptoethanol (Sigma Aldrich catalog) and 150 µl nano pure water.

For staining of gels with coomassie brilliant blue (described later), a volume of protein suspension equal to 35 µg protein was diluted to a volume of 20 µl with the appropriate buffer as noted throughout. For staining of gels using the silver salts (described later), a volume of protein suspension equal to 5 µg protein was diluted to a volume of 20 µl with the appropriate buffer as noted throughout. Where noted, denatured samples were heated for ten minutes at 90^{o}C in a Thermolyne type 17600 sandbath. 20 µl of the protein suspension was loaded into separate wells. Into remaining wells, 5 µl of BioRad AllBlue pre-stained protein standards were loaded or BioRad Laemmli sample buffer to serve as a visible dye front for tracking progress of the gel.

Gels were run using the BioRad ProteanII system (BioRad discontinued item) and a Tris-Glycine-SDS buffer (running buffer) containing, in one liter, 22.5 g Glycine (Fisher Scientific; FW 75.06), 4.5 g Tris and 1.0 g SDS. Gels were run at a constant voltage as noted throughout until the dye front reached the bottom of the gel. Gels were then removed from the apparatus for further use.

Larger format gels (16 cm) were poured by hand using the formulations found in Table 1.

Two dimensional gel electrophoresis

While SDS-PAGE is useful for separating proteins based on molecular weight, a more powerful technique, known as two dimensional gel electrophoresis (2D PAGE) is able to separate proteins in a first dimension based on isoelectric point (pI). Subsequent

Table 1: Formulations for large format SDS-PAGE Gels. All compounds except TEMED were mixed together and degassed by vaccuum for 10 minutes. Gels were immediately cast upon addition of TEMED. Resolving gel was overlayed with an equal parts mixture of butanol to 0.5M Tris-HCl (pH 6.8) buffer. 40% Acrylamide purchased from BioRad (catalog # 161-0144). TEMED (catalog # BP150-100) and ammonium persulfate (catalog # A-1433) purchased from Fisher Scientific.

	4% Stacking Gel	**7.5% Resolving Gel**	**10% Resolving Gel**	**12% Resolving Gel**
40 % Acrylamide	1.5 ml	11.25 ml	15 ml	18 ml
0.5 M Tris-HCl (pH 6.8)/1% SDS	3.75 ml	--	--	--
1.5 M Tris-HCl (pH 8.8)/1% SDS	--	15 ml	15 ml	15 ml
Nano pure water	9.50 ml	33.75	30 ml	27 ml
10% ammonium persulfate (prepared fresh)	300 µl	300 µl	300 µl	300 µl
TEMED	30 µl	30 µl	30 µl	30 µl

use of SDS-PAGE allows separation of proteins by molecular weight. This technique allows for greater resolution of discrete proteins. Here, 2D PAGE was used to investigate the proteome of OhILAs grown under varying conditions. Prior to 2D PAGE, samples underwent TCA precipitation as previously described. The remaining pellet was resuspended in 100 µl of 2D lysis buffer. Protein yield and concentration were determined as previously described.

A volume equal to the recommended amount of protein as noted throughout was diluted to either 125 µl (7cm strips) or 200 µl (11 cm strips) as noted in a buffer (rehydration buffer) containing 7M urea, 2M thiourea, 4% CHAPS, 10 mM DTT, a trace amount of bromophenol blue (Fisher Scientific) and 1/100 volume 100X Bio-Lyte 3-10 carrier ampholytes (20%) (BioRad). The bromophenol blue served as a dye front to track progress of the isoelectric focusing. Immobilized pH gradient (IPG) strips of pH range 3 to 10 were purchased from BioRad Laboratories in lengths of 7 cm (catalog # 163-2000) or 11 cm (catalog # 163-2014) as noted throughout. Isoelectric focusing was performed in the IEF Cell (catalog # 165-4000) from BioRad laboratories. Electrodes were covered w/ filter paper which was saturated with 10 µl of nano pure water. The sample was pipetted along the edge of the channel. The plastic covering was removed from the strip and the strip was then placed gel side down on top of the sample. The strips were covered with an appropriate volume of mineral oil. Rehydration and sample loading were done using active rehydration at 50 volts for 12 hours. Following rehydration, the strips were focused using the parameters as detailed in Table 2.

Following isoelectric focusing the strips were equilibrated for ten minutes in equilibration buffer 1 containing in 20 ml 6 M urea, 2% SDS, 0.375 M Tris-HCl (pH

Table 2: Isoelectric focusing guidelines for pH 3-10 strips. Adapted from ReadyStrip IPG Strip Instruction Manual

	7 cm	**11 cm**
Start Voltage	0 V	0 V
End Voltage	4,000 V	8,000 V
Volt-hours	10,000	35,000
Ramp	Rapid	Rapid
Temperature	20^{o} C	20^{o} C

8.8), 20 % glycerol and 2% DTT followed by equilibration for ten minutes in equilibration buffer 2 containing in 20 ml 6 M urea, 2% SDS, 0.375 M Tris-HCl (pH 8.8), 20 % glycerol, 2% DTT and 0.5 g iodoacetamide (Acros Organics; FW 184.96). The strips were then soaked in 100 ml of running buffer and transferred to a resolving gel (percent acrylamide specified throughout); either a 7 cm pre-cast or a large format 16 cm gel prepared as previously described. The strip was overlayed with overlay agarose of the following composition: 25 mM Tris, 192 mM glycine, 0.1 % SDS, 0.5% low melting point agarose (Fisher Scientific). Proteins were separated by molecular weight as previously described.

Staining of proteins using Coomassie Brilliant Blue R-250

To visualize proteins, gels containing >30 μg of protein per lane were stained using the Coomassie Brilliant Blue technique (Coomassie stained). Gels were soaked overnight in a dye solution containing in 500 ml 0.12 g brilliant blue (Fisher Scientific), 125 ml methanol (Fisher Scientific), 50 ml glacial acetic acid (Fisher Scientific) and brought to volume with nano pure water. Following the staining procedure, gels were soaked in a 10% glacial acetic acid solution until bands resolved. Addition of one or two KimWipes in the container speeded destaining procedure. Gels were imaged in Kodak 1D ver 3.5.4 LE software using a Kodak DC290 camera housed in a Kodak EDAS 290 housing. A Fisher Scientific light source (model # DLT-A) provided the lightsource. Unless otherwise noted, exposure times were 1/250 of a second. Gels were then dried for 3 hours (7 cm) or 5.5 hours (16 cm) at 60^{o}C with a slab gel dryer (model # SGD4050)

and gel pump (model # GP100) from Savant. Fresh calcium chloride (Fisher Scientific catalog; FW 110.99) was used as dessicant.

Staining of proteins using silver salts

To visualize proteins, gels containing < 5 μg of protein per lane were stained using the following silver stain protocol. Immediately following SDS-PAGE, gels were removed to a fresh solution of 40% methanol (Fisher Scientific) and 7.41% formaldehyde (Sigma Aldrich) in distilled water for twenty minutes (7cm) or one hour (16 cm). Gels were then thoroughly washed with distilled water before soaking in a 0.02% sodium thiosulfate (Sigma Aldrich; FW 158.1) solution for two minutes. Gels were washed two times for twenty seconds per wash. Gels were then soaked in a 0.1% silver nitrate (Sigma Aldrich; FW 169.9) solution for ten minutes. Gels were washed with water and with a small volume of developing solution containing in one liter 30 g sodium carbonate (Fisher Scientific; FW 105.99) 1.35 ml 37% formaldehyde and 4 mg of sodium thiosulfate. Gel was then placed in developing solution until faint bands began to appear. Addition of 5 ml 2.3 M citric acid (Fisher Scientific; FW 192.13) per 100 ml developing solution slowly stopped the developing reaction. Gels were then soaked in a drying solution of 30% ethanol and 4% glycerol in distilled water prior to imaging and drying as previously described.

In gel assay of arsenate reductase activity

To qualitatively assay for arsenate reductase activity, and in gel activity assay was conducted. Native SDS-PAGE gels were run as previously described. The gel was then

soaked in a reduced methyl viologen buffer containing 10 mM Tris-HCl (pH 8.0) and 5 mM methyl viologen (Sigma Aldrich; FW 257.17). The methyl viologen was reduced with 0.25 g of sodium dithionite (Sigma Aldrich; FW 174.1) to form a brilliant blue color. After the gel was sufficiently stained, the reduced methyl viologen solution was removed and the gel was washed in a 5 mM arsenate buffer containing 10 mM Tris-HCl (pH 8.0) and 5 mM sodium arsenate (Sigma Aldrich; FW 312.02) until clear bands began to appear where reduction was occurring. The gel was immediately imaged as previously described. The gel was subsequently coomassie stained, imaged and dried.

In vitro assay of arsenate reductase activity

To quantify the arsenate reductase activity present in the protein samples, an *in vitro* assay was performed. A MVr buffer solution containing 300 μM methyl viologen, 1.3 mM sodium arsenate and 10 mM Tris-HCl (pH 8.0) was degassed using argon (Airgas) and reduced with 50 μl of a buffer containing 0.575 M dithionite also under argon atmosphere. Starna Cell anaerobic 1.5 ml quartz cuvettes with a 1 cm pathlength were degassed with argon. 1 ml of the reduced 300 μM MV buffer was anaerobically transferred to the cuvette using a 3 ml syringe.

Native protein suspensions were diluted to a 1 μg/ μl concentration in lysis buffer 1. Caps were briefly removed and 5 μl of protein suspension (experimental) or lysis buffer 1 (control) were added to the cuvette, which was immediately recapped. Absorbance at 600 nm was monitored for ten minutes. A MVox buffer solution containing 300 μM methyl viologen, 1.3 mM sodium arsenate and 10 mM Tris-HCl (pH 8.0) was used as reference. As control, this method was repeated using arsenate free

MVr and MVox buffers. Activity curves were constructed as detailed in results. Specific activity was calculated from the linear portion of the curves using the equation

$$(((\Delta Abs./time)/\epsilon)/1000)*200$$

where the extinction coefficient for reduced methyl viologen is equal to 13.1 L $mole^{-1}$ cm^{-1}.

Western blot for ArrA1

As detailed by Thangavelu (2004), a synthetic peptide corresponding the the C-terminus of ArrA1 from *Bacillus selenitereducsens* (N-KCYGQGHWAYGHIAS-C) was used to raise polyclonal antibodies in two rabbits. The antibody, anti-ArrA1, was then affinity purified using ArrA1 as the ligand. The synthetic peptide from *B. selenitereducsens* has 73% identity to the C-terminus of OhILAs ArrA1 (Schaeffer et al., 2001)(Altschul et al., 1997).

To assay for the presence of ArrA1, a Western blot was conducted using a heat denatured SDS-PAGE gel with TCA precipitated samples. After the gel was run, a blotting sandwich was prepared by soaking the gel, two gel size pieces of Ahlstrom Filtration 0.83 mm filter paper (Fisher Scientific), a piece of GE Water & Process Technologies 0.45 micron NitroBind nitrocellulose membrane (Fisher Scientific) cut to gel size and two sponges in a transfer buffer containing in one L 14.4 g glycine, 3.03 g Tris and 100 ml methanol (pH 8.3) until saturated. Handling the nitrocellulose with forceps, the blotting sandwich was prepared. The sandwich was placed into a BioRad gel box and chamber was filled with transfer buffer. An ice pack and magnetic stir bar were used to cool set-up during transfer. Proteins were transferred at 250 mA for 30 minutes.

After transfer, gel was Coomassie stained as previously described. Nitrocellulose was incubated at ambient temperature in Killer Filler containing in one liter 5 g casein (Sigma Aldrich), 0.01 M sodium hydroxide (Fisher Scientific; FW 40.00), 900 ml phosphate buffered solution (appendix 1), 5 g bovine serum albumin (Sigma Aldrich), 0.1 g phenol red and 1.8 g sodium azide (Sigma Aldrich; FW 65.01) (pH 7.4) for two hours. The nitrocellulose membrane was then incubated overnight in a primary antibody solution containing anti-ArrA1 (appendix 1) affinity purified from rabbit serum in Killer Filler at a 1:1000 dilution. The membrane was washed twice in a TTBS solution containing in one liter 9 g sodium chloride, 6.05 g Tris-HCl (pH 7.5) and 0.5 ml Tween-20 (Fisher Scientific) followed by washing two times with a TBS solution containing 9 g sodium chloride and 6.05 g Tris-HCl (pH 7.5). The membrane was incubated for a minimum of two hours in secondary antibody (anti-Rabbit) and again washed twice each with TBS and TTBS.

The membrane was developed by combining solution 1 (7.5 mg 5-bromo-4-chloro-3-indolyl phosphate dissolved in 0.5 ml dimethyl formamide (Sigma Aldrich) with solution 2 (15 mg Nitro Blue tetrazolium (Sigma Aldrich catalog # N-6876; FW 817.6) dissolved in 0.35 ml dimethyl formamide and 0.15 ml distilled water) in 50 ml of carbonate buffer containing 8.4 g sodium bicarbonate and 0.2 g magnesium chloride in one liter (pH 9.8). The solution was poured over the membrane, which was allowed to develop for the specified time in darkness.

In vitro assay for sodium dependent transformation of roxarsone.

To develop an assay to monitor the disappearance of roxarsone *in vitro*, whole cell suspensions were obtained as previously described and suspended in a 10 mM Tris-HCl (pH 8.0) buffer. Anaerobic cuvettes containing 1 ml of a buffer containing 10 mM Tris-HCl (pH 8.0), 100 μM roxarsone, and the specified concentration of sodium chloride were degassed with hydrogen and sealed. 100 μl of the whole cell suspension was added to the reaction mixture and absorbance at 400 nm was recorded at the specified time points.

Upon completion, the 600 nm absorbance of each sample was recorded, and whole cells were removed from the sample using a 0.22 μm filter as previously detailed. A full-wavelength scan was then performed on the remaining filtrate. The data was graphed in GraphPad Prism 5.0.

Full wavelength scans of roxarsone, 2-nitrophenol and proposed metabolites

To develop a reliable activity spectrophotometric activity assay to monitor the disappearance of roxarsone and the structurally similar 2-nitrophenol, full wavelength scans of these compounds and the proposed metabolites, 3-A-4-HBAA and 2-aminophenol, were needed to establish peak absorbance. 1 mM stock solutions of the above mentioned compounds prepared in 10 mM Tris-HCl (pH 7.3) were diluted to 100 μM concentrations. A full wavelength scan was performed from 250 nm to 900 nm. The results were plotted in GraphPad Prism 5.0.

In vitro assay to assay concentration of 3-A-4-HBAA or 2-aminophenol

The primary metabolites expected from the transformation of roxarsone or the structurally similar 2-nitrophenol are 3-A-4-HBAA or 2-aminophenol respectively. Full wavelength spectra have demonstrated that 3-A-4-HBAA has minimal impact at the peak absorbance for roxarsone, 400 nm. However, full wavelength data obtained on 2-nitrophenol and 2-aminophenol indicate that there may be interference near the peak absorbance for the respective compounds. To accommodate for this, a colorimetric assay detecting amino compounds, development of an assay to monitor the concentration of both 3-A-4-HBAAs began. This method was adapted from the assay for aminobenzoate (Rafil et al., 1991)

To produce a standard curve for each of the compounds, 1 mM stock solutions of 3-A-4-HBAA and 2-aminophenol were prepared in 10 mM Tris-HCl (pH 8.0) buffer. The 1 mM stock solutions were diluted to either a 100, 50, 25 or 10 µM final concentration in 10 mM Tris-HCl buffer in a final volume of 1 ml. To this volume, 200 µl of 2.1% (w/v) trichloroacetic acid and 20 µl of 0.7% sodium nitrite were added. The reaction was incubated for twenty minutes at ambient temperature. 20 µl of 4% (w/v) ammonium sulfamate was added to the reaction. Following a 3 minute incubation at room temperature, 700 µl of 1% NEDD was added to the reaction, which was then incubated for 35 minutes. Addition of 60 µl 10 mM Tris-HCl (pH 8.0) brought the final volume to 2 ml.

Absorbance of the final reaction product at 543 nm was recorded (Rafil et al., 1991) and plotted against the concentration of the organoarsenical. Extinction coefficients for 3-A-4-HBAA (ε = 11.03 L mole^{-1} cm^{-1}) and 2-aminophenol (ε = 3.11 L

mole^{-1} cm^{-1}) were calculated from the slope of the standard curve. As control, the same procedure was followed starting with 1 mM stock solutions of either roxarsone or 1 mM 2-nitrophenol. Extinction coefficients associated with these compounds were negligible.

Results

Establishing the roxarsone extinction coefficient

To develop a spectrophotometric assay to monitor concentrations of 3-nitro-4-hydroxy benzene arsonic acid, a standard curve was established at 400 nm (Figure 7). It has been previously been established that roxarsone has a peak absorbance in the visible range, near 400 nm. Furthermore, it has also been previously established that the expected products, those of 3-A-4-HBAA and 4-hydroxybenzene arsonic acid, have minimal absorbance at this wavelength (Fisher, 2006).

A standard curve of roxarsone (Figure 1) was established using a one millimolar stock solution that was diluted to the following concentrations: 500, 250, 150, 100, 50, and 25 micromolar. Absorbance was recorded for the samples at 400 nm and the data was graphed in GraphPad Prism 5.0. An extinction coefficient (ε) was calculated from the slope of the linear regression ($\varepsilon = 2.841\ mM^{-1}cm^{-1}$). All calculations were performed in GraphPad Prism 5.0.

Aerobic chicken litter enrichments do not transform 3-nitro-4-hydroxybenzene arsenic acid

Previous evidence from our lab and others has shown that anaerobic cultures of chicken litter enrichments are capable of transforming roxarsone. However, there is no present study investigating the role aerobic cultures may play in this same phenomenon.

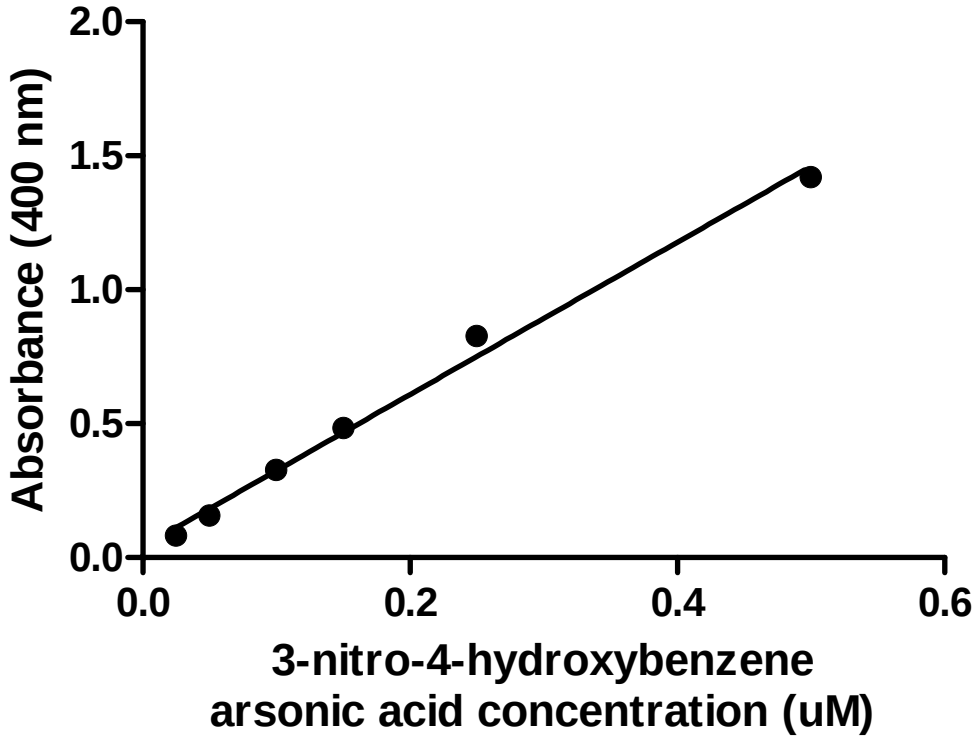


Figure 7: Standard curve of roxarsone concentration. A one millimolar stock solution was diluted to the following concentrations: 500, 250, 150, 100, 50, and 25 micromolar. Absorbance = 400 nm, ε = 2.841 $mM^{-1}cm^{-1}$ ($r^2$0.9927). Prepared in GraphPad Prism 5.0. Calculations performed using linear regression.

To investigate whether microbial cultures obtained from a chicken litter enrichment were capable of transforming roxarsone aerobically, enrichment cultures were grown on aerobic OhBs media using lactate as the initial carbon source. Cultures were inoculated with one milliliter of slurry and incubated at ambient temperature throughout the duration of the experiment.

Samples were collected initially and continued at intervals throughout the duration of the experiment. Aerobic cultures demonstrated growth as expected in the presence of 3-nitro-4-hydroxybenzene arsonic (1 mM) acid as well roxarsone (1 mM) and sodium lactate (10 mM) as measured spectrophotometrically at 600 nm (Figure 8). Understandably, there was an increased cell yield in cultures grown in the presence of lactate than in those grown in its absence (Figure 8). However, there was no transformation of roxarsone observed as determined by the spectrophotometric assay at 400 nm in either the lactate cultures or the cultures without lactate (Figure 8). These data suggest that the rapid transformation of roxarsone seen previously (Garbarino et al., 2003); (Morrison, 1969); (Gupta & Charles, 1999); (Rosal et al., 2005); (Rutherford et al., 2003) is likely the result of biotransformations occurring under anaerobic conditions. In contrast, Brown et al (2005) detected 5% transformation of roxarsone in cultures treated with Ap (top horizon) soil of the Frederick soil profile at pH 5.

Clostridium sp. strain OhILAs demonstrates increased growth in the presence of 3-nitro-4-hydroxy benzene arsonic acid.

Previous evidence has suggested that *Clostridium* sp. strain OhILAs is able to transform the organoarsenical 3-nitro-4-hydroxybenzenearsonic acid (roxarsone) when

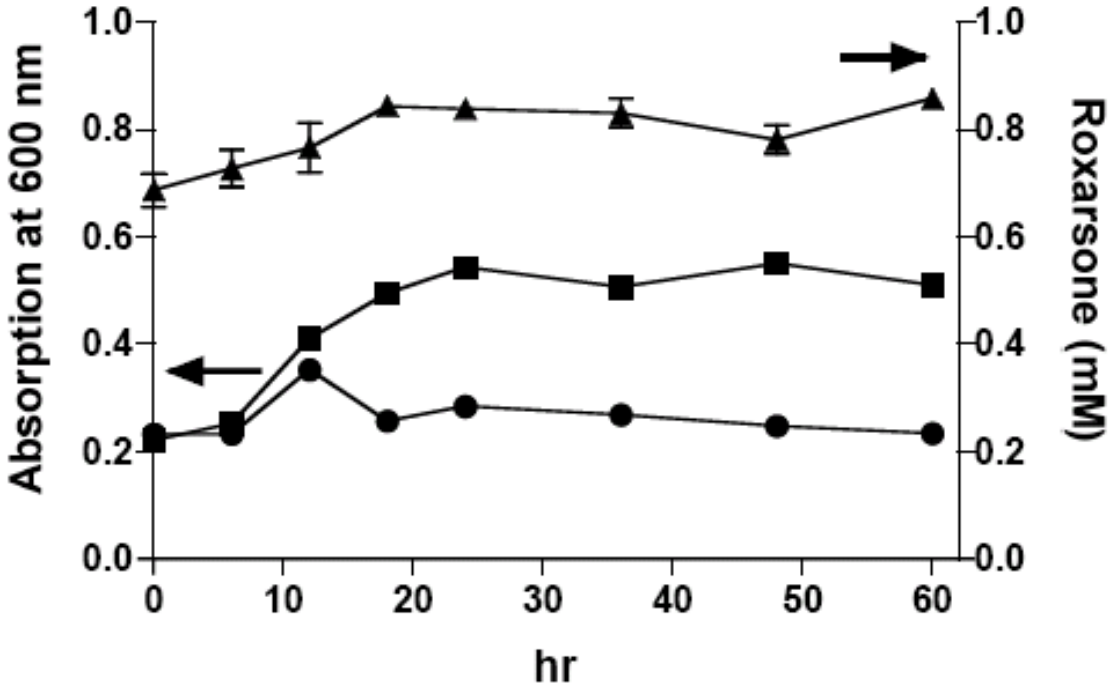


Figure 8: Aerobic growth of chicken litter enrichments in the presence of roxarsone. Growth (determined at 600 nm) is increased in the presence of lactate (■) compared to cultures grown in the presence of roxarsone alone (●). No change in roxarsone was seen as determined at 400 nm (▲). Aerobic OhBs media was prepared as detailed in "Materials and Methods" and amended to 1 mM roxarsone and, where indicated, 10 mM lactate. Concentration of roxarsone determined as described in "Materials and Methods" using $\varepsilon = 2.84\ mM^{-1}\ cm^{-1}$ as measured previously. Error bars represent standard error of the mean (SEM) as calculated from triplicate cultures.

grown anaerobically (Stolz et al 2007). The primary metabolite from this anaerobic transformation is a similar compound, 3-A-4-HBAA, where the nitro group on the third carbon has been reduced to an amine group (Stolz et al 2007). These results suggest that the organism may be using roxarsone as a terminal electron acceptor in anaerobic respiration.

To test whether OhILAs is capable of respiring roxarsone, cultures containing 10 mM of either fructose or lactate were grown in the presence or absence of roxarsone (1 mM) on OhBs media under a nitrogen atmosphere at ambient temperature. Samples were taken initially and at subsequent intervals throughout the growth phase of the organism to a final time point of 72 hours. The samples were analyzed spectrophotometrically at 600 nm for turbidity. Mid-log phase for the cultures was reached at approximately 48 hours for cultures grown on lactate in the presence of roxarsone and approximately 36 hours for those grown on fructose in the presence of roxarsone (Figure 9). The cultures grown in the absence of roxarsone did not reach mid-log phase in the 72 hour time span (Figure 9). These data indicate that OhILAs shows significantly increased growth when grown in the presence of roxarsone when compared to cultures grown in the absence of roxarsone ($p < 0.05$) (Figure 9). Additionally, visual colorimetric observation indicated that the roxarsone disappeared as the cultures were allowed to grow (data not shown). No growth or disappearance of roxarsone was seen in abiotic controls (data not shown).

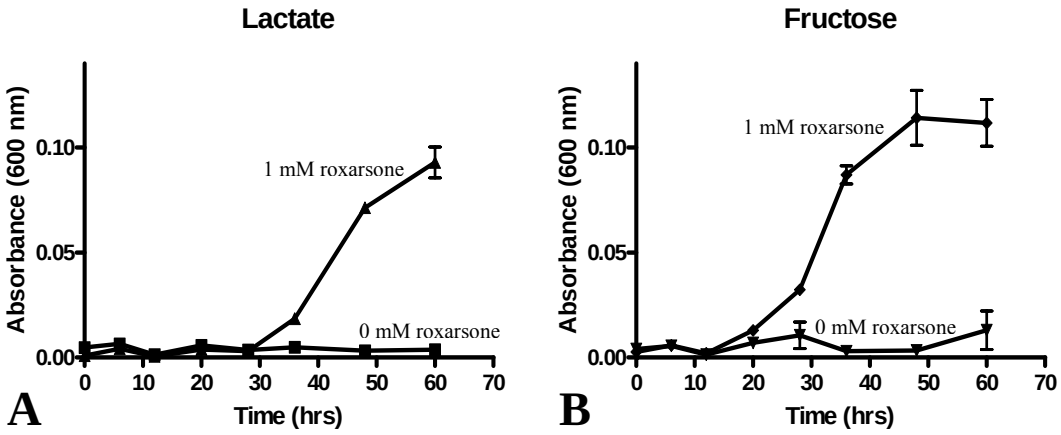


Figure 9: OhILAs demonstrates increased growth in presence of roxarsone. Cultures were grown on anaerobic OhBs media under an atmosphere of nitrogen at ambient temperatures in the presence (1 mM) or absence (0 mM) of roxarsone for either lactate (A) or fructose (B). Samples were taken initially and at time points indicated to a total of 72 hours. Means represent data obtained from three experimental cultures. No growth was seen in abiotic controls (not shown). Anaerobic OhBs media was prepared as detailed in "Materials and Methods" and amended to 1 mM roxarsone and, where indicated, 10 mM lactate or 10 mM fructose. Data plotted in GraphPad Prism 5.0. Error bars represent standard error of the mean (SEM) as calculated from triplicate cultures.

These data, coupled with the previous findings that OhILAs transforms roxarsone into 3-A-4-HBAA, suggest a role for roxarsone as a terminal electron acceptor during anaerobic respiration. Reducing equivalents generated during glycolysis are fed into the electron transport chain. These electrons generate an electrochemical gradient as they are passed along the chain, and are ultimately passed to the nitro group on the third carbon of roxarsone; subsequently reducing it to an amine group. This generates ATP via oxidative phosphorylation, resulting in increased growth of the organism.

Clostridium sp. strain OhILAs transforms the structurally similar 2-nitrophenol, but not 4-nitrophenol

As demonstrated previously, the nitro group on the third carbon of 3-nitro-4-hydroxybenzenearsonic acid (roxarsone) is reduced to an amine group producing the reduced compound, 3-A-4-HBAA acid (Stolz et al 2007). It is interesting to note that roxarsone, however, contains two oxidizing groups on its aromatic ring: the nitro group on the third carbon as well as the arsenate group on the first carbon. Evidence demonstrating OhILAs reduces only the nitro group suggests that the enzyme responsible for the initial reduction of a nitro to an amine is specific for a nitro group, and is not able to reduce the arsenate group.

To examine the specificity of the enzyme involved, transformation of the structurally similar compounds 2- and 4-nitrophenol was examined. Cultures of *Clostridium* sp. strain OhILAs grown on OhBs containing the presence (1 mM) or absence (0 mM) of one of the nitrophenolic compounds and/or the presence of lactate (10 mM) were grown under a nitrogen atmosphere at ambient conditions. Samples were

taken initially and every twelve hours thereafter for 72 hours. Samples were analyzed spectrophotometrically for turbidity at 600 nm. The 2-nitrophenol cultures reached mid-log phase prior to the 24 hour timepoint (Figure 10).

Transformation of 2-nitrophenol and 4-nitrophenol was monitored spectrophotometrically at the compound's peak absorbance; 410nm and 400nm respectively. Zeyer et al (1986) have previously established an extinction coefficient for 2-nitrophenol (ε = 3.47 $mM^{-1}cm^{-1}$ at 410 nm). From this, the concentration of 2-nitrophenol in solution was calculated using Beer's Law as previously detailed.

Consistent with the prediction that the enzyme responsible will reduce the nitro group located ortho to the hydroxyl group, the samples showed a corresponding decrease in 2-nitrophenol concentration (Figure 10) suggesting that the organism is capable of reducing the nitro group located in a position analogous to its location on the aromatic ring of roxarsone. Interestingly, the results of the same experiment conducted using 4-nitrophenol showed minimal transformation (data not shown).

Similarly, OhILAs grown on OhBs media amended with 10 mM lactate and 1 mM 2-nitrophenol show a similar physiological response to the nitro aromatic as the organism grown in the presence of roxarsone (Figure 11). Anaerobic cultures of OhILAs were grown in the presence of 10 mM lactate and either 1 mM 2-nitrophenol or 1 mM roxarsone. The absorbance at 600 nm or samples taken initially and at intervals throughout the experiment suggest that the physiological response of OhILAs grown in the presence of 2-nitrophenol is similar to OhILAs grown in the presence of roxarsone and provide further evidence to suggest that reduction of the nitro group is linked to respiratory processes.

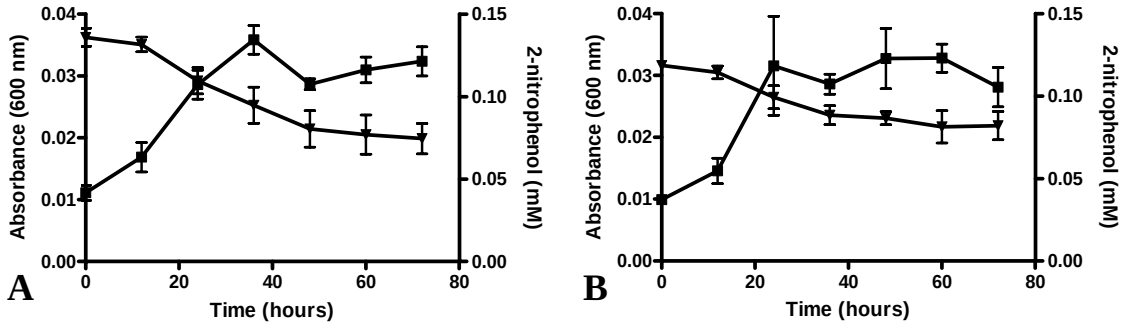


Figure 10: Growth curves of OhILAs in the presence or absence of lactate and 2-nitrophenol. Pure culture of OhILAs grown on OhBs amended to 1 mM 2-nitrophenol in the presence (A) or absence (B) of 10 mM lactate. Growth of OhILAs (■) as monitored at 600 nm coincides with disappearance of 2-nitrophenol (▼) concentration. Anaerobic OhBs media prepared as detailed in "Materials and Methods" and amended to 1 mM 2-nitrophenol and, where indicated, 10 mM lactate. Transformation monitored at 410 nm and converted to concentration using ε = 3.47 mM-1cm-1 (Zeyer et al., 1986). Error bars represent standard error of the mean (SEM) as calculated from triplicate cultures.

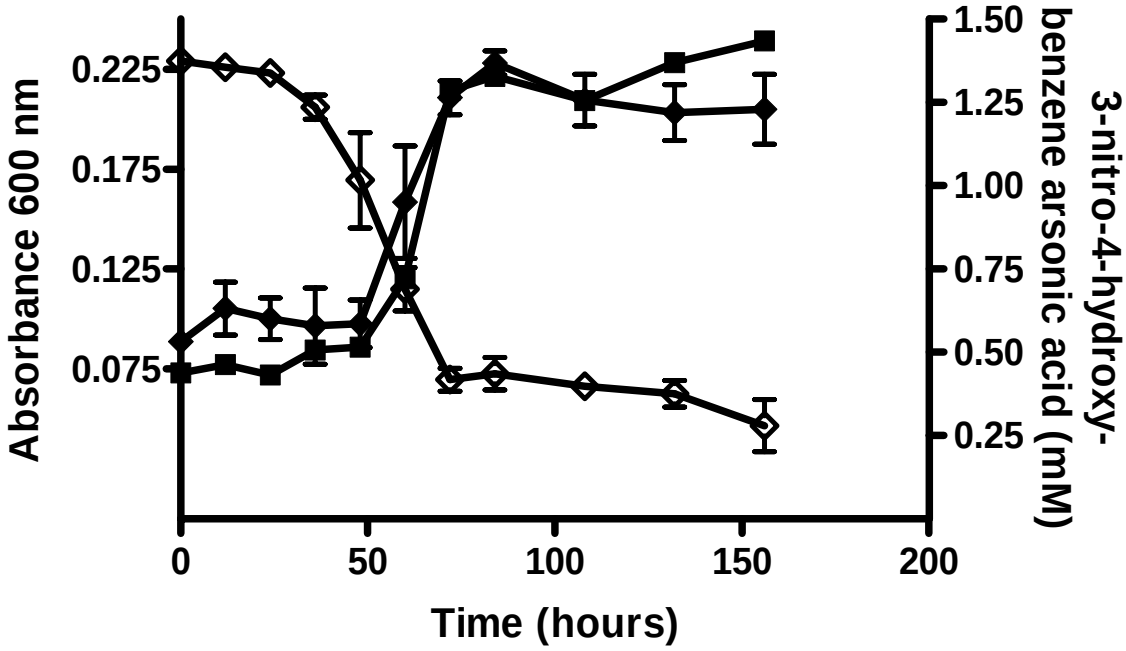


Figure 11: Physiological response – 2-nitrophenol. OhILAs grown in presence of 10 mM lactate and 1 mM 2-nitrophenol (■) is similar to OhILAs grown in presence of 10 mM lactate and 1 mM roxarsone (♦) as monitored at 600 nm. Roxarsone disappearance (◊) monitored at 400 nm and converted to mM concentration using $\varepsilon = 2.84\ mM^{-1}cm^{-1}$ as previously described. Anaerobic OhBs media was prepared as detailed in "Materials and Methods" and amended to 1 mM 2-nitrophenol or 1 mM roxarsone and 10 mM lactate. Data plotted in GraphPad Prism 5.0. Error bars represent standard error of the mean (SEM) as calculated from triplicate cultures.

Clostridium sp. strain OhILAs does not demonstrate increased growth in the presence of 3-A-4-HBAA or 4-hydroxybenzene arsonic acid

While the primary metabolite of roxarsone is 3-A-4-HBAA, it only accounts for about 70% of the roxarsone that disappears. The remaining metabolite composition can be accounted for with the production of arsenate and, to a lesser extent, arsenite. This suggests a number of possibilities involved in the mechanism, including further transformation of 3-A-4-HBAA.

To test whether OhILAs was capable of growing in the presence of intermediates in the proposed pathway of roxarsone breakdown, OhILAs was grown anaerobically in the presence of 10 mM lactate and 1 mM of either 3-A-4-HBAA or 4-hydroxybenzene arsonic acid (Figure 12). These data clearly demonstrate that OhILAs does not exhibit increased increased cell yield in the presence of these intermediaries. This might suggest that further transformation of 3-A-4-HBAA either does not occur and that the initial production of arsenate is directly from 3-nitro-4-hydroxy benzene arsonic acid, or that further transformation of 3-A-4-HBAA occurs through physiological or environmental changes brought about by the transformation of roxarsone.

Annotated OhILAs genome – genes of interest

The genome of this organism is nearing completion. With the most recent draft genome available, several key proteins were identified that are of interest in elucidating the mechanism behind the metabolic breakdown of roxarsone (Table 3). Among these, OhILAs contains sequences which potentially encode both the resistance arsenate reductase (*ars*) as well as the respiratory arsenate reductase (*arr*) pathway.

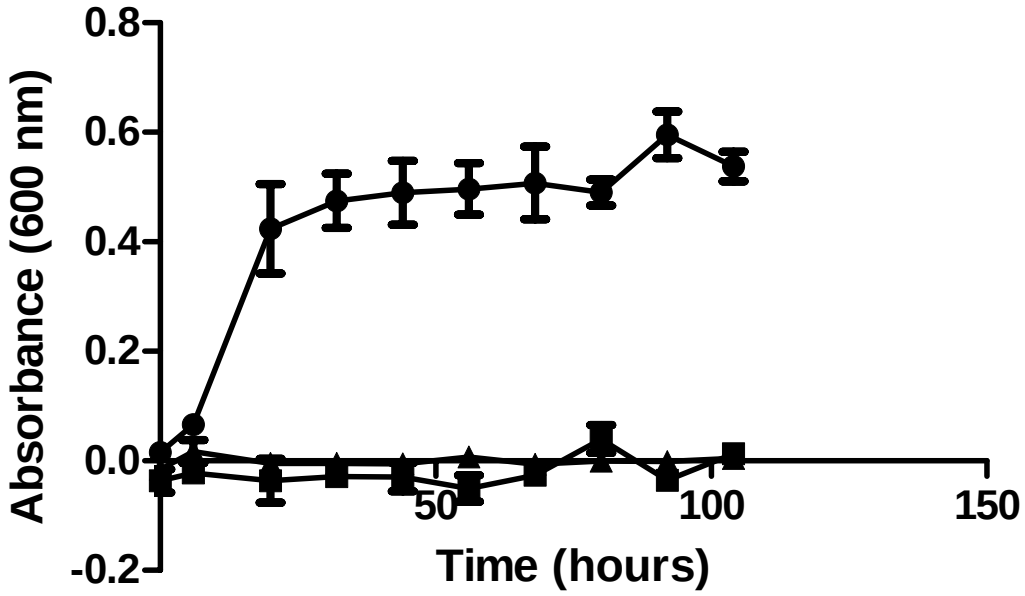


Figure 12: Growth of OhILAs in presence of proposed intermediates. OhILAs was grown anaerobically on OhBs media amended with 10 mM lactate and 1 mM of either roxarsone (●), 3-A-4-HBAA (■) or 4-hydroxybenzene arsonic acid (▲). Anaerobic OhBs media was prepared as detailed in "Materials and Methods" and amended to 1 mM of either 3-A-4-HBAA or 4-hydroxybenzene arsonic acid and 10 mM lactate. Growth represents absorbance at 600 nm. Data plotted in GraphPad Prism 5.0. Error bars represent standard error of the mean (SEM) as calculated from triplicate cultures.

Table 3: Potential protein targets for understanding the mechanism of roxarsone transformation. Molecular Weight (MW) and isoelectric point (PI) calculated using ExPASY suite.

	Contig	Gene	Function	MW	PI
***ars* Operon**			**Resistance**		
arsA	110	1081	ATP-ase	64515.47	5.24
arsB (acr3)	110	1085	AsIII pump	39260.95	8.34
arsC	110	1084	AsV reductase	14757.89	5.19
arsD	110	1080	regulatory	5108.07	8.61
arsR	110	1083	regulatory	14195.72	5.78
***arr* Operon**			Respiration		
arrB	110	1068	FeS	24877.08	5.60
arrA	110	1067	catalytic	99529.29	6.82
arrC	110	1066	anchoring	22436.11	5.54
arsM	110	1065	methylase	28353.28	5.08
PETN reductase	109		**Degradation**		
nemA		860	oxidoreductase	39346.71	5.48
nemA		891	xenobiotic reductase	37899.45	5.85
Nitroreductase			Nitroreduction		
	83	2219	nitroreductase	22980.53	6.24
	111	1447	nitroreductase	19250.29	7.01
	108	667	nitroreductase	26297.81	8.96
F0F1 ATPase			Respiration		
atpA	81	2183	alpha	55550.78	5.32
atpB	81	2181	beta	49939.99	4.90
atpG	81	2182	gamma	32320.81	8.94
atpH	81	2184	delta	20397.80	5.17
atpC	81	2180	epsilon	15155.29	5.58
atpI	81	2187	a	25271.27	7.95
atpF	81	2185	b	25271.27	7.95
atpE	81	2186	K	8752.42	5.11
Acrylate pathway					
lactate dehydrogenase (FMN Dependent)	110	1125		36029.88	6.96
Malate/lactate dehydrogenases	85	2263		46789.45	5.70
pyruvate-ferredoxin oxidoreductase 1	110	1014		66961.19	5.67
pyruvate-ferredoxin oxidoreductase 2	112	1563		64845.71	5.78
pyruvate-ferredoxin oxidoreductase 3	70	1984		65280.46	5.71
phosphotransacetylase	111	1309		32110.46	5.48
phosphotransacetylase	91	2438		32405.83	5.16
acetate kinase	97	2595		43234.64	5.55
Stickland Ferment					
Glycine reductase	74	2041		4555.30	4.72
Glycine reductase	74	2043		38101.30	5.00
Glycine reductase	74	2044		8250.76	6.73
Glycine reductase	90	2399		69036.25	5.04

Investigations into the activity of the arsenate reductase have suggested that OhILAs might maintain constitutive expression of these proteins. Using reduced methyl viologen as an electron donor, a colorimetric in vitro activity assay was conducted. Oxidation of methyl viologen was monitored spectrophotometrically in the presence or absence of arsenate as an electron acceptor. The data demonstrate that under all growth conditions, both soluble and insoluble fractions were capable of oxidizing the methyl viologen in the presence of arsenate, but not in its absence.

In gel arsenate reductase assay suggests arsenate reductase is constitutively expressed

Ultimately, the concern lies with the production of inorganic arsenic from the transformation of roxarsone. As mentioned earlier, the primary metabolite is 3-A-4-HBAA, though the remaining mass can be accounted for with production of arsenate and, to a lesser extent, arsenite. Previous evidence demonstrates that the initial form of inorganic arsenic is in the form of arsenate, and that later there is production of arsenite. This suggests that the production of arsenite is from respiration of arsenate.

The annotated genome of OhILAs reveals genes encoding the components of the respiratory arsenate reductase pathway, as well as those coding for the resistance pathway discussed previously. In gel activity assays using reduced methyl viologen as an electron donor were used to qualitatively assay for expression of arsenate reductase. Methyl viologen is a useful indicator that changes color between reduced and oxidized forms. Reduced methyl viologen is blue in color. When the reduced methyl viologen donates two electrons to arsenate, forming arsenite, the oxidized methyl viologen is clear in color.

A buffer containing 1 mM methyl viologen in 10 mM Tris-HCl (pH 8.0) was reduced with 0.25 g sodium dithionite. A native 12% SDS-PAGE gel (Figure 13) containing 35 μg soluble (1-4) and insoluble (5-8) OhILAs protein fraction grown on 10 mM lactate (1,5) or 10 mM lactate and 1 mM of either roxarsone (2,6), 2-nitrophenol (3,7) or or 5 mM sodium arsenate (4,8).

The gel was run as described earlier and stained in the 1 mM methyl viologen solution reduced with sodium dithionite for fifteen minutes. The methyl viologen solution was removed, and the gel was then washed with a 5 mM sodium arsenate solution in 10 mM Tris-HCl (pH 8.0) buffer. An approximately 75 kDa protein band in the gel showed observable clearings in all fractions (Figure 13) when washed with the sodium arsenate solution. This suggests that an enzyme present was transferring electrons from the reduced methyl viologen to the arsenate (forming arsenite). Since methyl viologen autooxidizes, the gel was imaged immediately (Figure 13) as previously detailed.

In vitro arsenate reductase assay suggests arsenate reductase is constitutively expressed

The results of the in gel assay suggest that arsenate reductase is constitutively expressed. To quantify the specific activity in the samples, protein fractions were diluted to a 1 μg/μl suspension. A buffer containing 300 μM methyl lviologen, 1.3 mM sodium arsenate and 10 mM Tris-HCl (pH 8.0) under argon was reduced with sodium dithionite under argon. One milliliter was anaerobically transferred to an argon degassed cuvette. Five micrograms of protein was added to the reaction mixture by quickly removing the

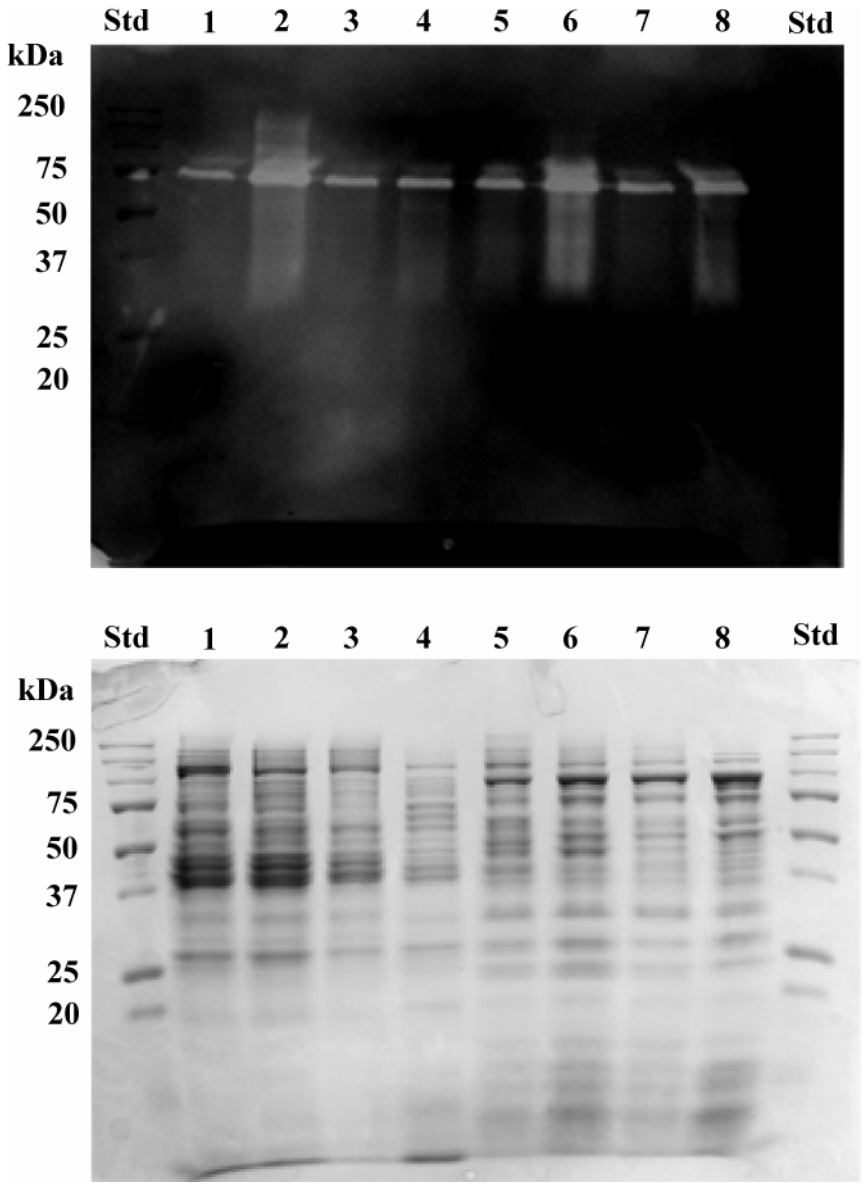


Figure 13: Arsenate reductase in gel assay. 12% native gel stained with 1 mM reduced methyl viologen in 10 mM Tris-HCl (pH 8.0) and washed with 5 mM sodium arsenate in Tris-HCl (pH 8.0) (A) shows oxidation of reduced methyl viologen (clearing) in both soluble (1-4) and insoluble (5-8) fractions of OhILAs grown on 10 mM lactate (1,5) or 10 mM lactate and 1 mM of either roxarsone (2,6), 2-nitrophenol (3,7) or or 5 mM sodium arsenate (4,8). (B) Gel from A after Coomassie staining showing that clearing is associated with approximately 75 kDa band.

cap and pipetting in the required volume. Using the oxidized methyl viologen buffer as reference, the oxidation of methyl viologen was followed at 600 nm Specific activity was calculated using $\varepsilon = 13.1$ for reduced methyl viologen.

Activity was observed in cytosolic (soluble) (Figure 14) OhILAs protein fraction grown on 10 mM lactate (Figure 14A) or 10 mM lactate and 1 mM of either roxarsone (Figure 14B), 2-nitrophenol (Figure 14C) or or 5 mM sodium arsenate (Figure 14D). Additionally, activitity was also observed in the membrane, or insoluble, fraction grown on 10 mM lactate (Figure 15A) or 10 mM lactate and 1 mM of either roxarsone (Figure 15B), 2-nitrophenol (Figure 15C) or 5 mM sodium arsenate (Figure 15D). No activity was observed in samples where a 300 μM methyl viologen buffer in 10 mM Tris-HCl (pH 8.) was reduced with sodium dithionite without the presence of arsenate (data not shown). This suggests that an enzyme is indeed involved in oxidation of methyl viologen by transferring two electrons to arsenate and forming arsenite. arsenate to arsenite.

Though enzymes generally tend to be specific for a particular substrate, it is not outside the realm of possibility that, with an oxidizer and a reducer present in such high concentrations, there could be non-specific transfer of electrons by promiscuous enzymes. To test whether ArrA1 was present in the samples, the non-denatured fractions were run in a 12% acrylamide gel. Proteins from the gel were then transferred to a nitrocellulose filter which was incubated in a blocking solution and then in a 1:1,000 dilution of primary antibody rabbit anti-ArrA1 overnight. Following incubation in primary antibody, the membrane was washed and incubated using a 1:5,000 dilution of the secondary goat anti-rabbit antibody and developed using the alkaline phosphatase

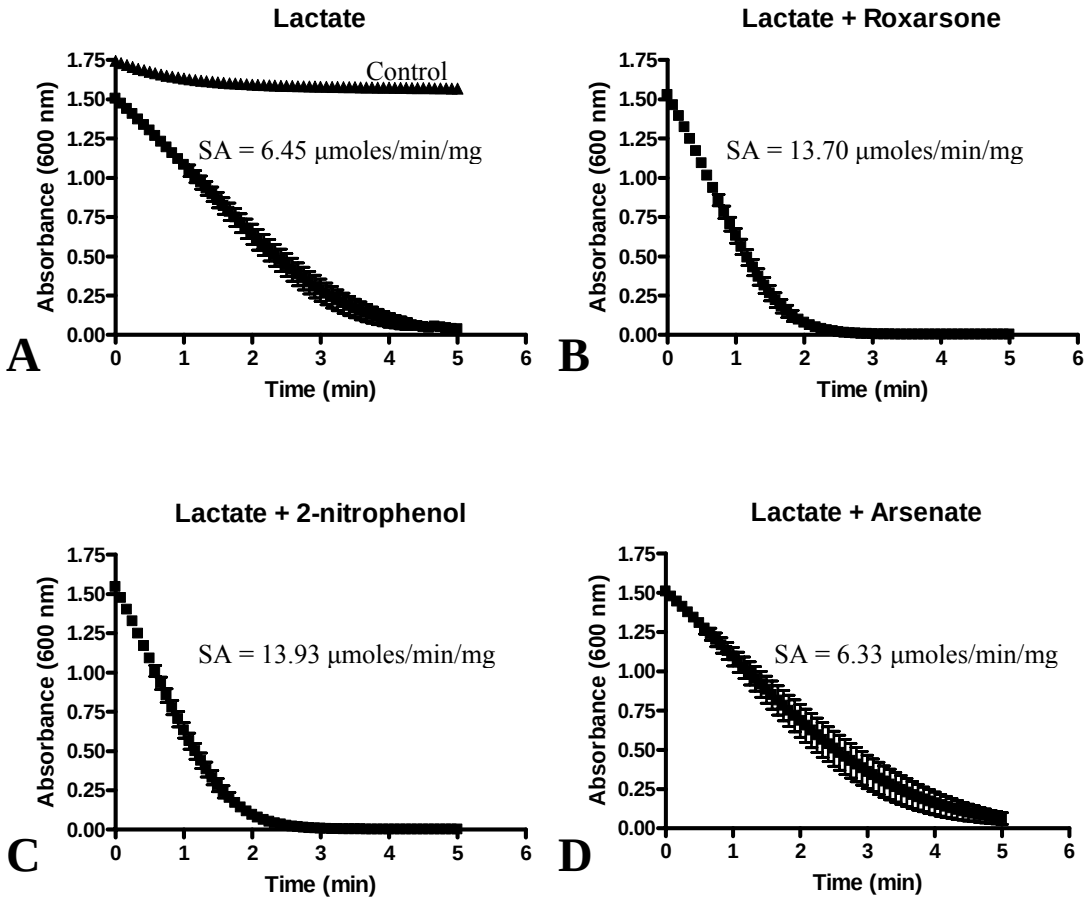


Figure 14: Activity of cytosolic (soluble) fractions. OhILAs cytosolic protein fractions grown in the presence of 10 mM lactate (A) or 10 mM lactate and 1 mM of either roxarsone (B), 2-nitrophenol (C) or 5 mM sodium arsenate (D). 5 µl of a 1 µg/µl protein suspension was added to 1 ml of a 300 µM methyl viologen and 100 mM Tris-HCl (pH 8.0) in the presence (experimental) or absence (control) of 1.3 mM sodium arsenate buffer reduced with sodium dithionite. Specific activity calculated at an absorbance of 600 nm as previously detailed using $\varepsilon = 13.1\ mM^{-1}\ cm^{-1}$ for. Data plotted in GraphPad Prism 5.0. Error bars represent standard error of the mean (SEM) as calculated from triplicate cultures.

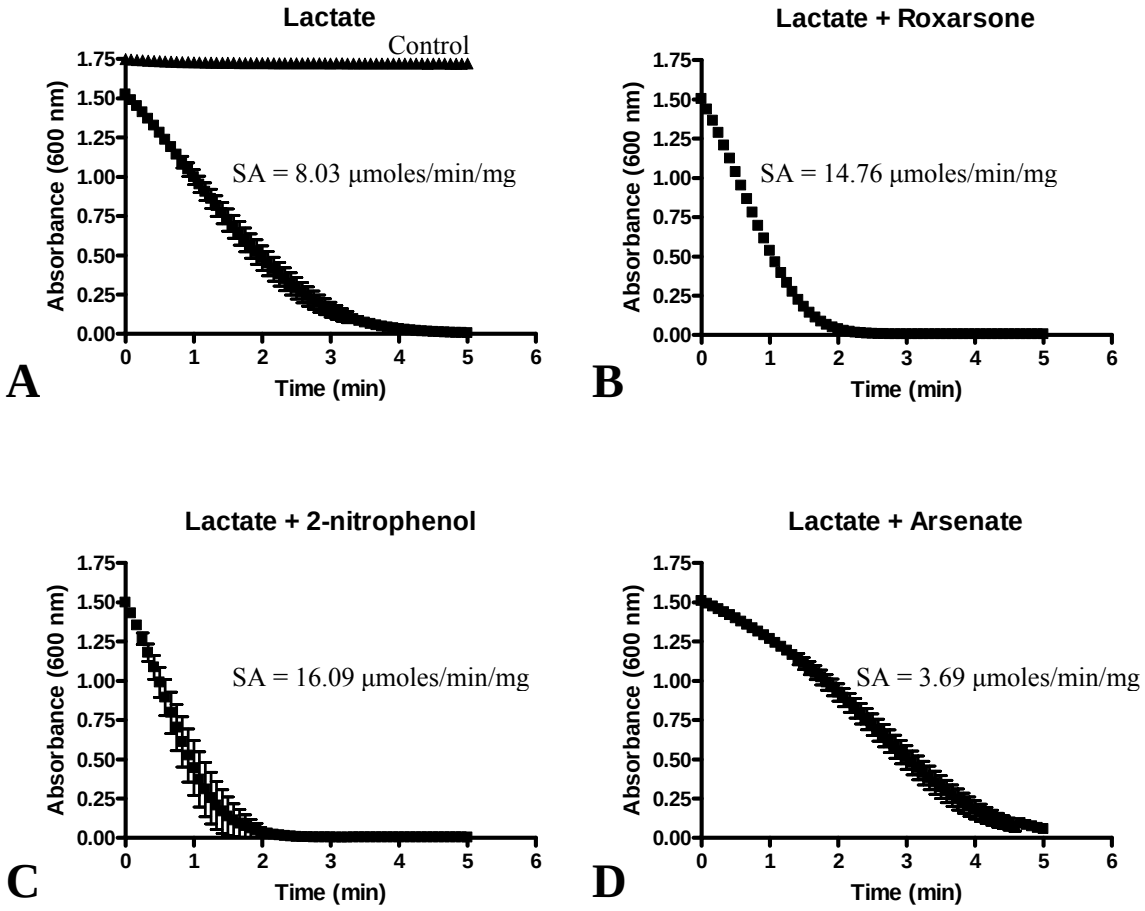


Figure 15: Activity of membrane (insoluble) fractions. OhILAs soluble protein fractions grown in the presence of 10 mM lactate (A) or 10 mM lactate and 1 mM of either roxarsone (B), 2-nitrophenol (C) or 5 mM sodium arsenate (D). 5 µl of a 1 µg/µl protein suspension was added to 1 ml of a 300 µM methyl viologen and 100 mM Tris-HCl (pH 8.0) in the presence (experimental) or absence (control) of 1.3 mM sodium arsenate buffer reduced with sodium dithionite. Specific activity calculated at an absorbance of 600 nm as previously detailed using $\varepsilon = 13.1\ mM^{-1}\ cm^{-1}$ for. Data plotted in GraphPad Prism 5.0. Error bars represent standard error of the mean (SEM) as calculated from triplicate cultures.

reaction. Results of this Western blot (Figure 16) provide evidence to suggest that OhILAs ArrA1 expression is constitutive.

In vitro assay of roxarsone transformation

The annotated OhILAs genome suggests that it encodes several target proteins of interest that may be involved in the transformation of roxarsone. Among these proteins, OhILAs contains a region coding for a F_1/F_0 sodium dependent ATP synthase. Imkamp & Mueller (2002) demonstrated that reduction of caffeate by the bacterium *Acetobacter woodii* was sodium dependent. Here, preliminary development of an *in vitro* activity assay to determine if transformation of roxarsone is sodium dependent is discussed.

Full wavelength scans of 3-nitro-4-hydroxybenzene arsonic, 2-nitrophenol, and their proposed metabolites were performed using 100 μM concentrations in 10 mM Tris-HCl (pH 7.3) (Figure 17). This data was useful in interpreting the results of subsequent activity assays.

Interestingly, results of the full wavelength spectral data (Figure 17) suggest that monitoring 2-nitrophenol disappearance directly using spectrophotometry is undesirable. While 2-nitrophenol has a broad peak absorbance around 410 nm. However, consistent with the results of Gonçalves et al (2000) the spectral data interference at this wavelength. It is suggested that the interference is the result of quinine formation as suggested by Gonçalves and colleagues (2000). To compensate for this, an assay measuring the concentration of amine compounds was developed using modification of an existing protocol (Rafil et al., 1991). A standard curve of 3-A-4-HBAA and 2-aminophenol were prepared.

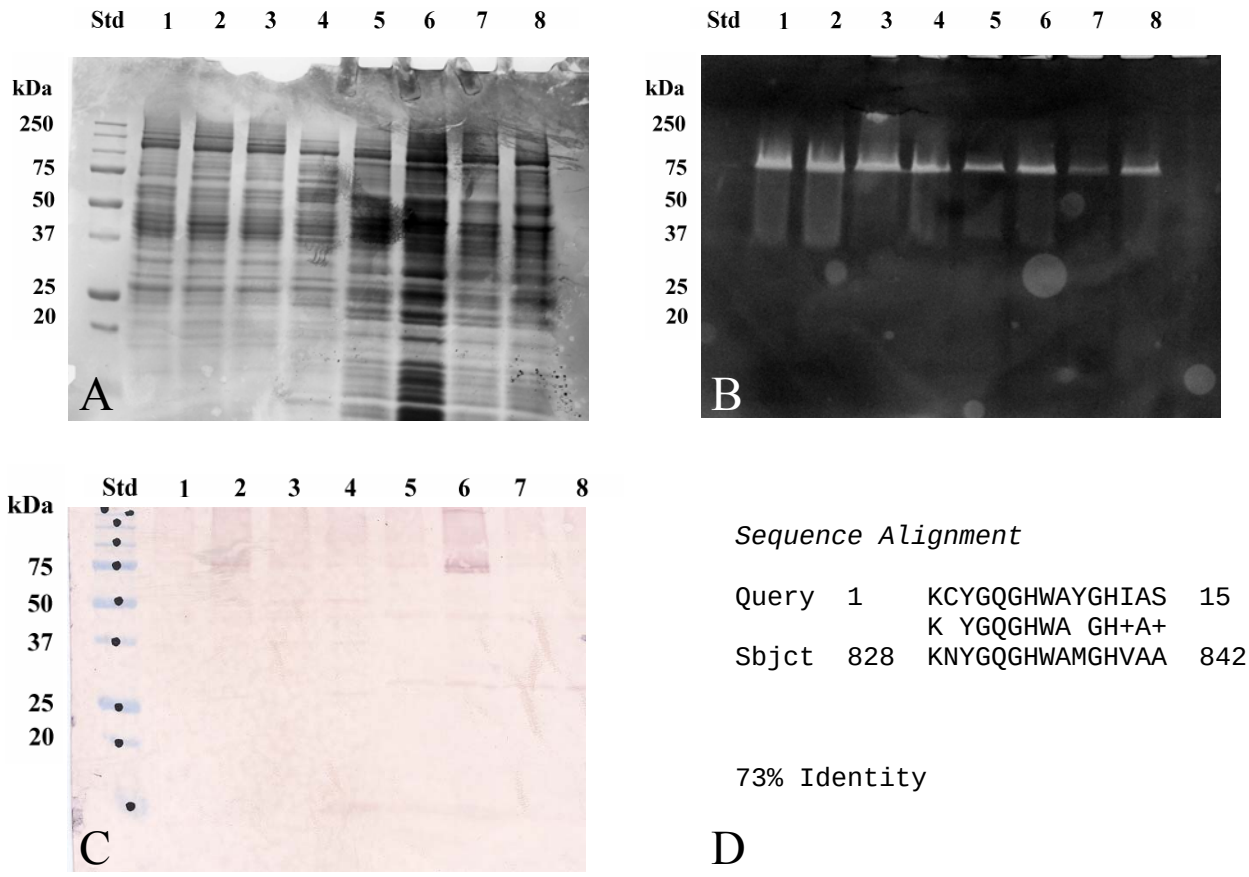


Figure 16: Western blot of ArrA. Native SDS-PAGE gel (A) indicated that protein fractions of OhILAs (1-4) and membrane (5-8) OhILAs protein fractions grown on 10 mM lactate (1,5) or 10 mM lactate and either 1 mM roxarsone (2,6), 1 mM 2-nitrophenol (3,7) or 5 mM sodium arsenate (4,8) were consistently loaded and positive for arsenate reductase activity (B). A native gel was transferred to nitrocellulose membrane and blotted using anti-ArrA1 rabbit primary antibody and anti-rabbit goat secondary antibody and developed using alkaline phosphatase reaction as detailed in "Materials and Methods" (C). Polyclonal anti-ArrA1 were raised in a rabbit system against a synthetic peptide (Query) corresponding to *Bacillus selenitereducsens* ArrA1 C-terminus (Thangavelu, 2004) which has 73% identity to a fifteen peptide stretch of OhILAs ArrA (Sbjct) (D) (Schaeffer et al., 2001)(Altschul et al., 1997).

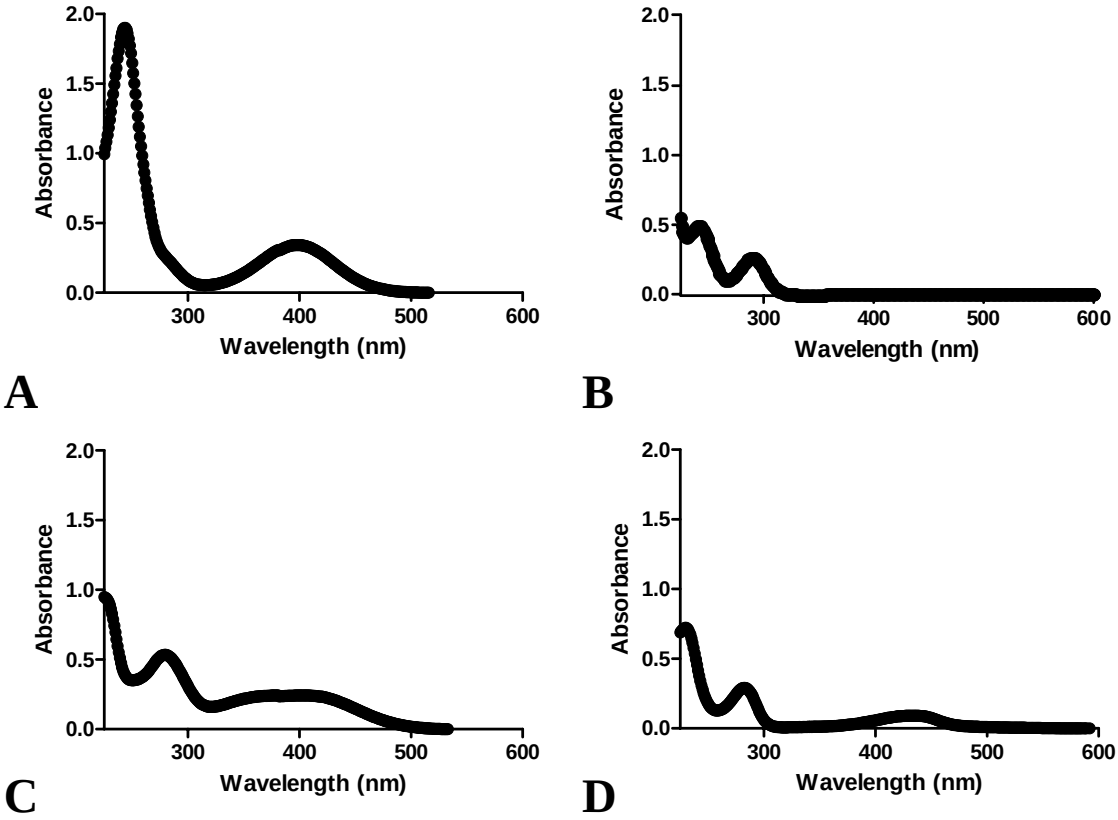


Figure 17: Full wavelength spectra of roxarsone, 2-nitrophenol, 3-amino-4-hydroxybenzene arsonic acid and 2-aminophenol. 100 μM concentrations of roxarsone (A), 2-nitrophenol, (C) and the proposed metabolites 3-A-4-HBAA (B) and 2-aminophenol (D) were prepared in 10 mM Tris-HCl (pH 7.3). Data plotted in GraphPad Prism 5.0.

To produce a standard curve for each of the compounds, the previously described method was applied to 3-A-4-HBAA and 2-aminophenol. Assay specific extinction coefficients for 3-A-4-HBAA (ε = 11.03 L $mole^{-1}$ cm^{-1}) and 2-aminophenol (ε = 3.11 L $mole^{-1}$ cm^{-1}) were calculated from the slope of the standard curve (Figure 18). As control, the same procedure was followed starting with either roxarsone or 1 mM 2-nitrophenol. Extinction coefficients associated with these compounds were negligible (data not shown).

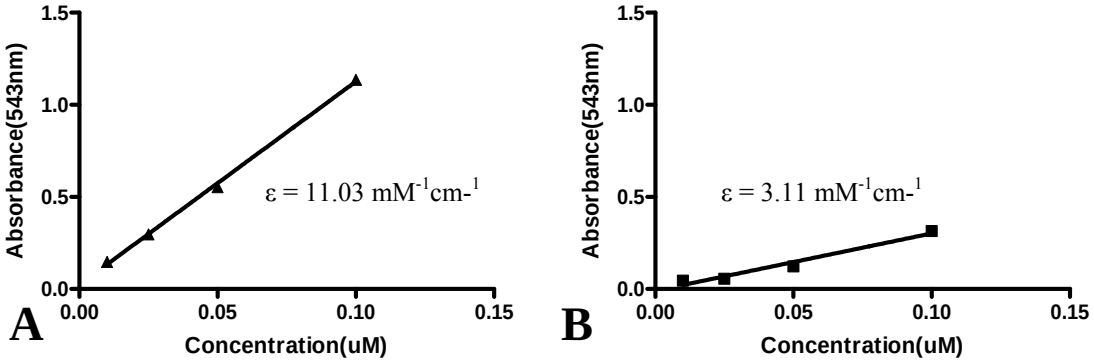


Figure 18: Standard curve of amino-organic concentrations prepared as described previously (Materials & Methods). Extinction coefficients (ε) for 3-A-4-HBAA (A) and 2-aminophenol (B) calculated from slope.

Discussion

The agricultural revolution has had vast cultural and economic impacts. Society has moved from an age where hunter-gatherers existed on what little was available in the forests to an age where acres of fields grow lush with corn, rice and various other agricultural staples. Instead of relying on hunting, with ensuing cycles of feast and famine, society is now able to rely on a constant supply of beef, poultry and pork products. There is no argument that this revolution has changed society for the better, ensuring better quality and more nutritious diets around the globe. However, from use of toxic pesticides and herbicides to hormonal supplements and feed additives, the United States agricultural industry has seen a much wider breadth of questionable compounds used in farming. While the use of some of these compounds may pose no environmental or human health risks, it is unsafe and impractical to use compounds until their mechanism of action as well as their behavior when released into the environment is fully understood.

Roxarsone, a feed additive used widely by the poultry industry for growing larger birds with better coloration (Abdo, 1989) is a compound that has come under recent investigation. The FDA considers this compound benign in its native state (Salsbury Laboratories, 1981), in part because it passes through chickens largely untransformed (Anderson & Chamblee, 2001). However, increased levels of arsenic found in fields treated with litter from chickens fed chicken feed containing roxarsone (Brown et al., 2005), have prompted further investigation into its behavior when released into the environment.

Recent evidence has suggested that roxarsone is rapidly transformed to a structurally similar organoarsenical, 3-A-4-HBAA (Stolz et al., 2007); (Cortinas et al., 2006). Since this compound is also used in the poultry industry and has long been considered benign, more pressing concerns arise when considering the remaining metabolites; compounds such as arsenate and, to a lesser extent, arsenite (Cortinas et al., 2006); (Stolz et al., 2007). While an exact mechanism of roxarsone transformation has yet to be agreed on, the previously mentioned evidence suggests that reduction of the nitro group to an amine may be the initial step, followed by successive breakdown, either aerobically or anaerobically, of the resulting 3-A-4-HBAA (Figure 5). This study investigates the role of chicken litter enrichment cultures and a pure strain *Clostridium* sp. strain OhILAs in the biotransformation of these compounds. Additionally, investigations into the proteome of OhILAs have revealed proteins that may be of interest in understanding the metabolic pathway involved in breakdown of roxarsone.

Previous research has suggested that the dominant organisms in anaerobic chicken litter enrichment cultures are clostridial species. Only spore-forming rods were observed using light microscopy of the enrichment cultures. Furthermore, limited diversity of 16s rRNA would suggests that clostridia are the dominant organisms of anaerobic chicken litter enrichments. This present study examines the physiology of roxarsone transformation by a clostridial species, OhILAs, as well as the ability of chicken litter enrichments grown aerobically to transform this organoarsenical.

Physiological roles of roxarsone transformation

The pattern of roxarsone transformation by OhILAs is similar to that observed previously in anaerobic chicken litter enrichment cultures. Initially, formation of 3-A-4-HBAA is observed, followed by release of inorganic species. While the observed initial times for these products differes between cultures of OhILAs and the enrichment cultures, it is imperative to remain cognizant of the fact that the enrichment cultures are mixed cultures, which could delay transformation of roxarsone. Nonetheless, the pattern of transformation between the two cultures remains very similar under these conditions. This observation allows further investigation into the physiology of roxarsone transformation by OhILAs. As detailed by Stolz et al (2007), reduction of the nitro group to an amine requires six electrons. This would suggest the following equation:

$$2 \text{ roxarsone} + 3 \text{ lactate} \rightarrow 2 \text{ 3-A-4-HBAA} + 3 \text{ acetate} + 3\ CO_2 + H_2O$$

In anaerobic transformation of roxarsone by both OhILAs as well as anaerobic chicken litter enrichments, formation of acetate was observed and was balanced with consumption of lactate. Traditionally, there are two common pathways in which lactate is fermented to yield acetate and propionate. In the succinate-propionate pathway (Figure 19), three molecules of lactate are oxidized to form three molecules of pyruvate by lactate dehydrogenase. This reaction results in the release of six electrons ([H]). The molecules of pyruvate are then further oxidized to form either acetate or propionate at a ratio of one molecule acetate to two molecules propionate. Conversion of one molecule

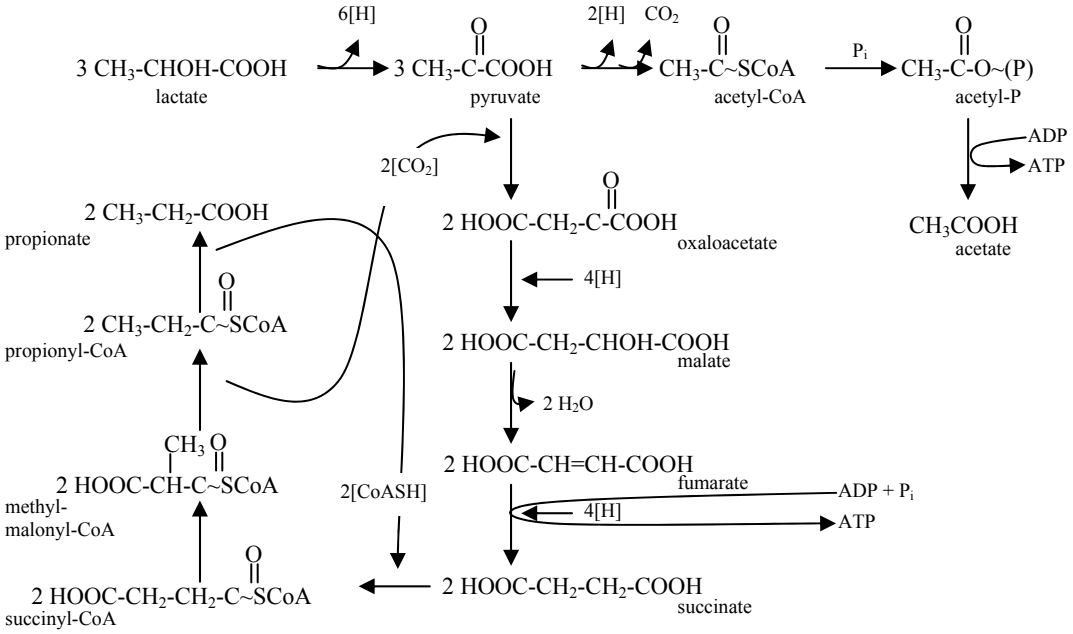


Figure 19: Succinate-Propionate Pathway (White, 1995).

of pyruvate to acetyl-CoA by pyruvate dehydrogenase results in the release of two additional [H]. This acetyl-CoA is then passed through phosphotransacetylase and acetate kinase to yield one molecule of acetate (White, 1995).

The two remaining molecules of pyruvate are converted to oxaloacetate by methylmalonyl-CoA-pyruvate transcarboxylase, a reaction which consumes two [H] per molecule of pyruvate. The oxaloacetates are then converted to malate by malate dehydrogenase followed by conversion to fumarate by fumarase. Consumption of an additional two [H] per molecule occurs in the reduction of fumarate to succinate via fumarate reductase. The two molecules of succinate are then converted are converted to two molecules of succinyl-CoA (CoA transferase) and subsequently methylmalonyl-CoA (methylmalonyl-CoA racemase). The resulting methylmalonyl-CoA is then converted to proprionyl-CoA when the carboxyl groups are donated to pyruvate via methylmalonyl-CoA-pyruvate transcarboxylase. The CoA group of proprionyl-CoA is donated to succinate by the CoA transferase resulting in formation of propionate (White, 1995).

Genomic analysis has indicated that certain components of this pathway were missing. Crucial among these are malate dehydrogenase and fumarate hydratase. Without these components, this metabolic pathway is unable to proceed from the oxaloacetate. Conversely, analysis of the genome has indicated that all necessary components of the acrylate pathway are present.

In the acrylate pathway (Figure 20), three molecules of latate are oxidized to form one molecule of acetate. Initially, one molecule of lactate is reduced to one molecule of pyruvate via the enzyme lactate dehydrogenase. This reaction results in the release of

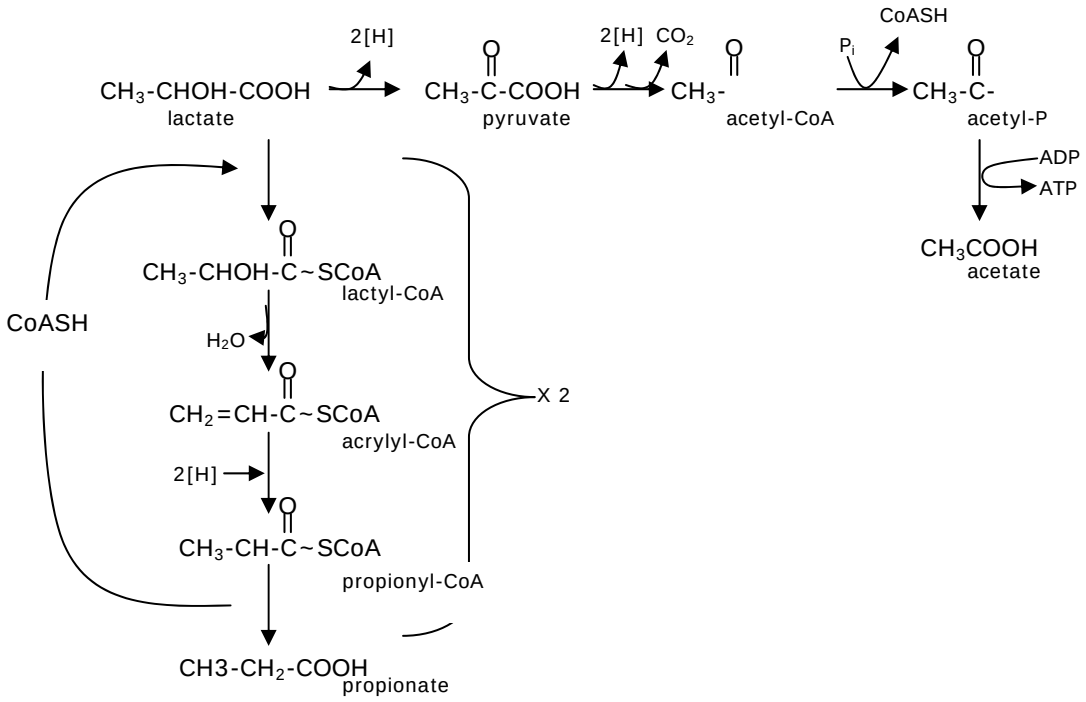


Figure 20: Acrylate Pathway (White, 1995).

two [H] per molecule of pyruvate produced. The pyruvate is further oxidized to acetyl-CoA with the release of carbon dioxide and two additional [H] via pyruvate-ferredoxin oxidoreductase. Ultimately, one molecule of acetyl-CoA produces one molecule of ATP via substrate level phosphorylation as well as production of one molecule acetate. The production of ATP and acetate from acetyl-CoA occurs via a complex two-step process. Initially, phosphotransacetylase converts acetyl-CoA to acetyl-P by addition of an inorganic phosphate and removal of CoASH. This intermediary is then converted to acetate via acetate kinase with the net result of formation of one molecule of ATP (White, 1995).

During fermentation, the reducing equivalents produced during this pathway are then passed to additional lactate molecules which have been converted to lactyl-CoA using a donated CoA group from proprionyl-CoA and the enzyme CoA transferase. Lactyl-CoA is dehydrated to form acrylyl-CoA by an uncharacterized reaction. The acrylyl-CoA is then reduced by the addition of two electrons to form proprionyl-CoA. As previously mentioned, proprionyl-CoA donates CoASH via CoA transferase to form lactyl-CoA and propionate. Unlike the components of the succinate-propionate pathway, all components of the acrylate pathway have been identified in OhILAs (White, 1995).

While clostridia are traditionally considered to have a fermentative metabolism, anaerobic respiratory processes cannot be negated. Recently, Imkamp & Mueller (2002) have described production of ATP coupled to the reduction of caffeate. The evidence suggests that this reduction occurs via a sodium (Na^+) dependent F_1/F_0 ATPase. Genomic of analysis of OhILAs has identified homologs of a Na^+ F_1/F_0 ATPase, NADH dehydrogenase and hydrogenase that would provide necessary components for an

anaerobic respiratory process (Stolz et al., 2007). These data, coupled with the previous results in which formation acetate was observed without detectable formation of propionate, suggest that the reducing equivalents produced from the initial oxidation of lactate to acetate may be shuttled to an electron transport system ultimately using roxarsone as the terminal electron acceptor to form 3-A-4-HBAA.

Further supporting this idea are the data obtained with respect to growth rates of OhILAs in the presence of roxarsone. Results of pure culture experiments demonstrated a significantly ($p < 0.05$) higher cell yield in cultures grown in the presence of 1 mM roxarsone and either 10 mM lactate or fructose when compared to cultures grown in the presence of lactate or fructose alone. In the case of aerobic respiration, electrons are passed from reducing equivalents, generated during glycolysis, such as reduced nicotinamide adenine dinucleotide (NADH) along the electron transport chain. As previously discussed, oxygen serves as the terminal electron acceptor in this mode of respiration and its reduction to water is ultimately coupled with the generation of copious amounts of ATP. In contrast, subsequent to glycolysis, organisms grown in cultures lacking an electron sink must generate ATP from fermentation, a process that produces vastly less amounts of ATP (Alberts et al., 2002).

Since the high energy phosphate bonds serve as the energy currency of the cell, a logical argument is made that less ATP yields less growth at a slower rate. Carried over, organisms grown fermentatively would have much slower growth with a much lower cell yield than those grown in the presence of a terminal electron acceptor. Indeed, this is what is seen in the cultures grown in the presence of roxarsone and either lactate or fructose as the initial carbon source and electron donor. Using previously obtained data

(Fisher, 2006), a standard curve of absorbance versus cells/ml (direct cell count) was plotted. Using the conversion factor ($2.98 * 10^7$), generation times during log phase growth of the cultures were calculated to be 2.2 hours in cultures grown in the presence of roxarsone and lactate while no generation time could be calculated for cultures grown in the presence of lactate alone. Similar results were obtained for cultures grown in the presence of roxarsone and fructose, where the generation time is 6.2 hours compared to an incalculable generation time for cultures grown on fructose alone. Moreover, at 60 hours, the cultures grown in the presence of roxarsone had a nearly ten-fold higher cell yield when compared to the cultures lacking roxarsone.

These results also suggest that a similar process may be occurring in chicken litter enrichment cultures. The pattern of transformant production observed in anaerobic experiments using chicken litter enrichments is similar to the patter observed by Stolz and colleagues (2007) in pure cultures. Anaerobic chicken litter enrichment cultures rapidly transformed roxarsone to 3-A-4-HBAA initially, followed by release of inorganic arsenic species (Stolz et al., 2007).

Conversely, while aerobic enrichment cultures still showed growth in the presence of roxarsone, a presumed antimicrobial, no detectable transformation of roxarsone was observed. These data make to interesting suggestions. Firstly, despite roxarsone being marketed as an antimicrobial, the microbial community of the chicken cecum is well suited to growing in the presence of roxarsone. Secondly, and perhaps more interestingly, it further support this them of roxarsone serving as a terminal electron acceptor.

In the presence of oxygen, lactate would have acted as an electron donor. The electrons generated from metabolic pathways such as the acrylate pathway would have been shuttled into an electron transport chain yielding high amounts of ATP but ultimately reducing oxygen to water. Conversely, when grown in the absence of oxygen, rapid transformation of roxarsone is seen coupled to increased cell yield.

The anaerobic culture results are consistent with, and expand on, the work of Cortinas and colleagues (2006). Here, a pure strain of *Clostridia* was used to monitor the transformation whereas Cortinas and colleagues (2006) examined transformation of roxarsone in non-specific microbial communities obtained from sewage sludge enrichment cultures. While the composition of the microbial community in the Cortinas study remains unresolved, it has been previously established that *Clostridia* are the dominant species of the chicken cecum. Additionally, these results are consistent with results suggesting that roxarsone is degraded during composting and storage of the chicken litter under anaerobic conditions (Garbarino et al., 2003).

Interestingly, these results are not consistent with findings by Brown and colleagues (2005) which suggest that aerobic enrichment cultures obtained from soil transform roxarsone at a level of 5%. This difference is possibly attributed to the experimental design. Whereas Brown and colleagues examined aerobic transformation of roxarsone using enrichment cultures obtained from soil, the present study is examining the transformation of enrichment cultures obtained from chicken litter. Furthermore, Brown and colleagues conducted their experiments at a pH of 5.0 whereas cultures involved in the present study are circumneutral at pH 7.3.

Physiological roles of 2-nitrophenol transformation

Clostridia species are well known for nitroreduction of aromatic compounds. Here, the evidence suggests an initial reduction of roxarsone to 3-A-4-HBAA followed by further degradation to inorganic arsenic species via pathways as yet uncharacterized. Furthermore, the evidence would suggest that roxarsone plays a role in anaerobic respiration as a terminal electron acceptor. To examine the role the arsenic group might play in initial reduction of the nitro group, OhILAs cultures were grown in the presence of the structurally similar 2-nitrophenol. The structure of roxarsone consists of an aromatic ring with a nitro group located ortho to the 4' hydroxyl group. Structurally, this is very similar to 2-nitrophenol. This compound consists of an aromatic ring with a nitro group located ortho to a hydroxyl group. Logically, reduction of the nitro group located ortho to the hydroxyl group should occur independently of the presence of an arsenate group on the aromatic ring. Additionally, if reducing equivalents generated during the acrylate or similar pathway are shuttled to an electron transport chain with roxarsone serving as the terminal electron acceptor, 2-nitrophenol should fulfill this role.

Here, anaerobic cultures of OhILAs were grown in the presence of 2-nitrophenol alone, or 2-nitrophenol and lactate. Incomplete transformation of 2-nitrophenol was observed as determined by a spectrophotometric assay. However, this is attributed to production of 2-aminophenol. The full-wavelength profile of the proposed transformation product, 2-aminophenol, shows some interference at 410 nm. Development of an azo dye based assay should clarify this situation.

It is interesting to note, however, that while 2-nitrophenol transformation occurred at detectable levels, transformation of 4-nitrophenol did not. Anaerobic cultures of 4-

aminophenol grown in the presence or absence of lactate or fructose yielded no increase in cell yield over cultures grown in the presence of the carbon source only. Moreover, no detectable level of transformation was observed. This suggests the substrate specificity of the enzymes involved in this pathway, and is consistent with results obtained with *Trichosporon* sp. Results have suggested that *Trichosporon* sp. isolated from the Po river in Italy are capable of degrading 2-nitrophenol but not 4-nitrophenol. It is also interesting to note that the organisms are capable of degrading 2,4-dinitrophenol, a compound not yet tested with OhILAs (Caselli & Hanau, 1994).

OhILAs growth response to addition of 3-A-4-HBAA or 4-hydroxybenzene arsonic acid.

As mentioned previously, the primary metabolite of roxarsone transformation by OhILAs is 3-A-4-HBAA, however, the remaining mass balance can be accounted for with compounds of much greater concern: arsenate and, to a lesser extent, arsenite (Stolz et al., 2007). While no pathway has been agreed upon, a proposed pathway suggests roxarsone is initially reduced to 3-A-4-HBAA and then there is additional degradation of this reduced organoarsenical. For this to occur, growth of OhILAs should still occur in the presence of the 3-A-4-HBAA as well as the proposed intermediate, 4-hydroxybenzene arsonic acid. Cultures grown in the presence of lactate and of the designated organoarsenical showed minimal cell yield over the duration of a 102 hour experiment.

The results of Cortinas and colleagues (2006) suggest that ultimately there is complete transformation of 3-A-4-HBAA, though it is a slow process. Prokaryotes have evolved several ways that amino groups can be removed from amino acids. While 3-A-

4-HBAA is not an amino acid, the compound may undergo deamination via a similar pathway. Typically, deamination of amino acids occurs by oxidatively deaminating the acid to its corresponding keto group. The keto groups are typically then converted to pyruvate and fed into the citric acid cycle (White, 1995).

Additionally, certain deamination pathways produce additional reducing equivalents. One such pathway is deamination via NAD(P)+-linked dehydrogenases. These enzymes reversibly aminate the keto acid and ultimately result in the production of ammonium and NADH (White, 1995). It may be possible that a non-specific NAD(P)+-linked dehydrogenase is involved in deamination of 3-A-4-HBAA. If occurring, the reducing equivalents produced may be involved in release of arsenic from the aromatic ring. At present, production of the proposed 3-A-4-HBAA transformant 4-hydroxybenzene arsonic acid has not been detected under laboratory conditions.

However, strict adherence to the above pathway would result in incomplete transformation of 3-A-4-HBAA. Two electrons would be necessary to reduce the arsenate group attached to the aromatic ring to an arsenite group and release it from the aromatic ring. Deamination by this pathway alone produces one reducing equivalent per amino group removed, suggesting that two molecules of 3-A-4-HBAA would be required for the release of one molecule of arsenite. Furthermore, it is proposed that inorganic arsenic is released in the form of arsenate and is then subsequently reduced to arsenite via respiratory mechanisms. Nonetheless, an adaptation of the above mentioned deamination mechanism may hold promise in understanding the release of inorganic arsenic species over time without observing production of 4-hydroxybenzene arsonic acid. Further

analysis of the OhILAs genome should prove fruitful in understanding this critical step in the degradation pathway.

To be certain, this is not the only such pathway that is possible. *Pseudomonas* sp. strain AP-3 utilizes a modified *meta*-cleavage pathway to metabolize aminophenols (Figure 21A) whereby the hydroxyl group located *ortho* to the amine is oxidized to form 2-aminomuconic acid via 2-aminomuconic 6-semialdehyde. Release of carbon dioxide to form 2-oxopent-4-enoic acid and addition of water ultimately forms 4-hydroxy-2-oxovaleric acid. A modified pathway is suggested in the transformation of 4-amino-3-hydroxybenzoic acid by *Bordetella* sp. strain 10d (Figure 21B) whereby 4-amino-3-hydroxybenzoic acid is ultimately converted to pyruvate and acetaldehyde via 2-amino-5-carboxymuconic 6-semialdehyde, 2-hydroxy-5-carboxymuconic 6-semialdehyde, 2-hydroxymuconic 6-semialdehyde, 2-hydroxymuconic acid, 4-oxalocrotonic acid, 2-oxopent-4-enoic acid and 4-hydroxy-2-oxovaleric acid (Orii et al., 2006). However, the annotated genome does not indicate any of the necessary components for either of these transformation pathways.

The OhILAs genome

As previously discussed, there are a wide array of arsenate resistance operons. The best studied is the operon on plasmid R773, which contains in order *arsR, arsD, arsA, arsB* and *arsC*, all under the control of a single operator/promoter region. While the minimum requirement for a functioning arsenate resistance system is *arsC*, all known operons contain at least three of these genes. However, it has been well documented that

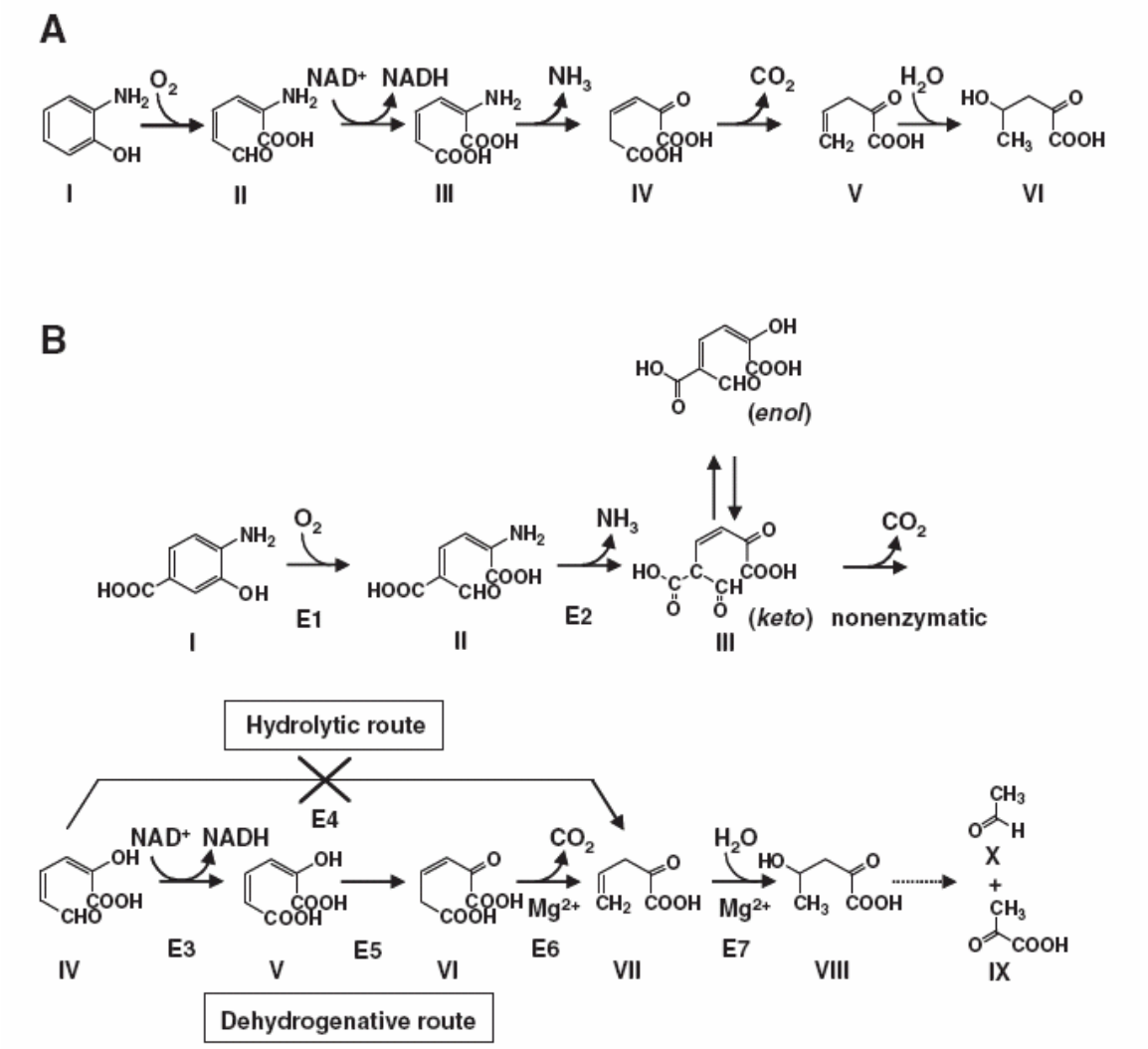


Figure 21: Aminophenol metabolism in *Pseudomonas* and *Bordetella* sp. Modified *meta*-cleavage of 2-aminophenol by *Pseudomonas* sp. strain AP-3 (A). The hydroxyl group located *ortho* to the 2-aminophenol (I) amine is oxidized to form 2-aminomuconic acid (III) via 2-aminomuconic 6-semialdehyde (II). Deamination to form 4-oxalocrotonic acid (IV) followed by release of carbon dioxide to form 2-oxopent-4-enoic (V) acid and addition of water ultimately forms 4-hydroxy-2-oxovaleric acid (V). Proposed metabolic pathway (B) of 4-amino-3-hydroxybenzoic acid (I) suggests that the compound is ultimately transformed to pyruvate (IX) and acetaldehyde (X) via 2-amino-5-carboxymuconic 6-semialdehyde (II), 2-hydroxy-5-carboxymuconic 6-semialdehyde (III), 2-hydroxymuconic 6-semialdehyde (IV), 2-hydroxymuconic acid (V), 4-oxalocrotonic acid (VI), 2-oxopent-4-enoic acid (VII) and 4-hydroxy-2-oxovaleric acid (VIII) (Orii et al., 2006).

the five gene operon (*arsRDABC*) encodes a much more effective arsenate resistance mechanism than three gene operons, typically *arsRBC* (Silver et al., 2002). The annotated genome reveals that OhILAs contains an intact five gene arsenate resistance operon.

Much more poorly understood is the mechanism behind arsenate reduction coupled to respiration. Intriguingly, OhILAs has a respiratory arsenate reductase (*arr*) consisting of *arrB, arrA, arrC* and *arsM*. This is a surprising result since both mechanisms transform arsenate to arsenite; however, previous evidence has shown that OhILAs is capable of growing in the presence of 40 mM arsenate, but only 10 mM arsenite (Fisher, 2006). Further investigations into the physiology of OhILAs in the presence of arsenate and arsenite, as well as a better understood characterization of the *arr* mechanism will aid in understanding these interesting results.

Previous research has indicated that *arrA* can be a reliable marker for environmental arsenate respiration. Between several phylogenetically diverse organisms, there was 61-100% conservation at the amino acid (Malasarn et al., 2004). Though OhILAs *arrA* does not share such high homology with other arsenate reductases, it does have 53% identity with *Chrysiogenes arsenatis* respiratory arsenate reductase (appendix 1) (Schaeffer et al., 2001)(Altschul et al., 1997). This enzyme has previously been purified and characterized (Krafft & Macy, 1998).

C. arsenatis respiratory reductase is a heterodimer consisting of an 87 kDa subunit (ArrA) and a 29 kDa subunit (ArrB) with a native molecular determined at 123 kDa that is localized in the periplasm. Several possible cofactors were identified for this enzyme. Among these, it is suggested that the enzyme may contain several iron sulfur

cluster prosthetic groups as well as a molybdenum cofactor. Zinc may also be a possible cofactor. The enzyme reduces arsenate to arsentite at neutral pH according to the following stochiometry:

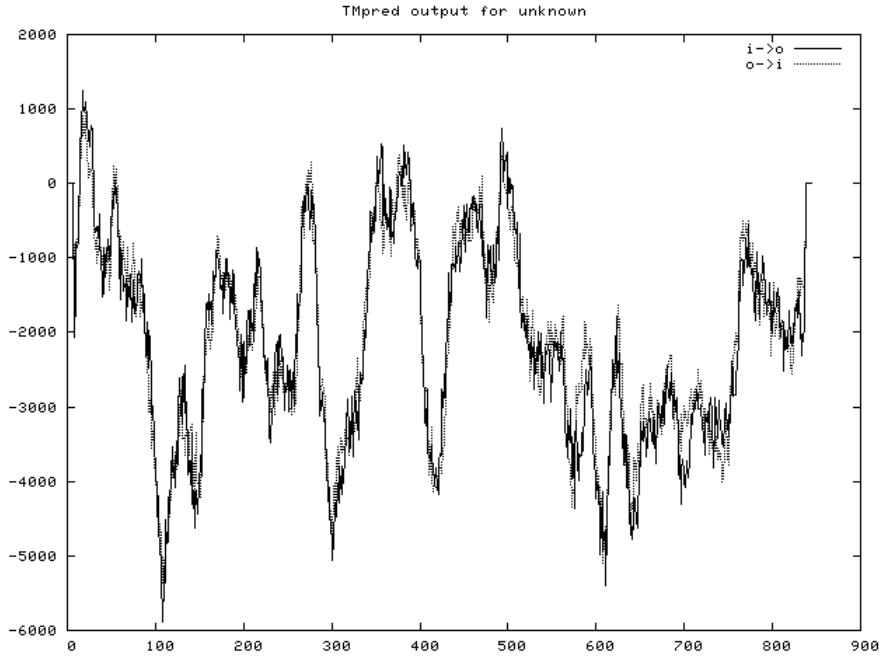
$$2\ HAsO_4^{2-} + 2\ H_2AsO_4^{-} + 5\ H^{+} + CH_3COO^{-} \rightarrow 4\ H_3AsO_3 + 2\ HCO_3^{-}$$

The arsenate reductase of *C. arsenatis* acts as the terminal reductase in anaerobic respiration. This suggests that, though the enzyme is freely soluble in the periplasm, *in vivo* it remains linked to a membrane bound electron transport chain (Krafft & Macy, 1998).

Topological analysis of these enzymes sequences suggest that they may contain transmembrane regions that would anchor the protein to the membrane. *C. arsenatis* ArrA is predicted to have five inside to outside transmembrane helices and eight outside to inside helices (Figure 22). The ArrA enzyme is predicted to have three inside to outside transmembrane helices and four outside to inside transmembrane helices (Hofmann & Stoffel, 1993). This suggests both enzymes may be capable of anchoring themselves to the periplasmic membrane. More intriguingly, the annotated genome suggests that the OhILAs arr operon encodes arrC, a 22.5 kDa anchoring protein.

These results could contribute to the inconsistent results obtained here with cytosolic and membrane fractions of OhILAs. *In vitro*, as well as in gel activity assays suggested that arsenate reductase activity was constitutive across the samples. However, no localization pattern was able to be determined from the results since all fractions tested positive for arsenate reductase activity. One possible explanation for this is that under non-denaturing conditions, a certain percentage of the ArrA subunit would have

A



B

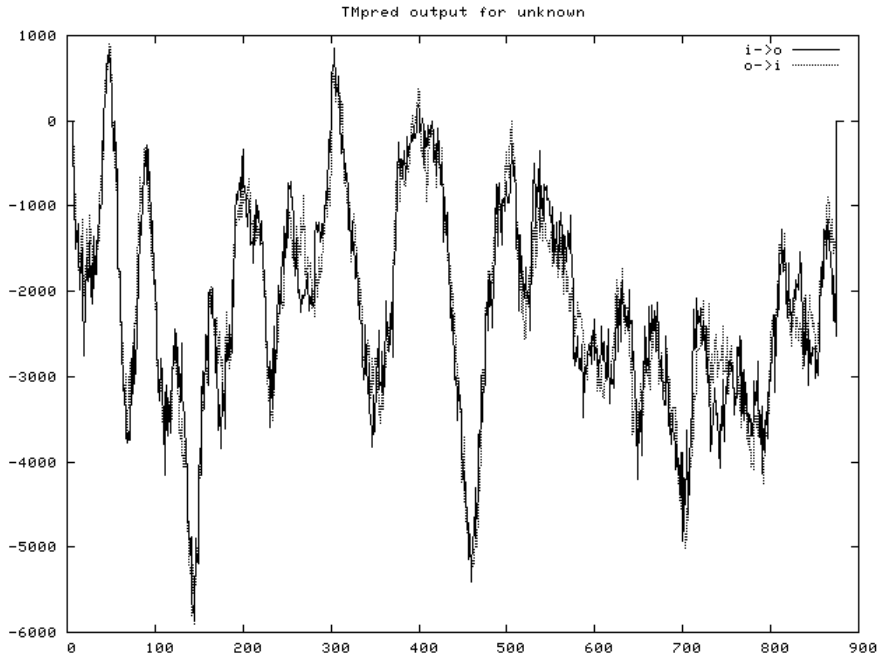


Figure 22: Topological prediction of ArrA from ***Chrysiogenes arsenatis*** (A) and OhILAs (B)**.** Topological prediction performed using TMpred. http://www.ch.embnet.org/software/TMPRED_form.html

remained attached to ArrC, and consequently activity would have been observed in the membrane fractions. Addition of the reductant dithiothreitol, however, would have caused a certain percentage of the enzyme to release from the membrane bound anchor and would have produced activity in the soluble fraction.

Another possibility exists, however. While this assay suggests that expression of an arsenate reduction enzyme may be constitutively expressed, it must be noted that both oxidizers (arsenate) and reducers (methyl viologen) were present in very high concentrations. In conditions such as these, it is not an unreasonable situation where a reductase, acting as a promiscuous enzyme, would non-specifically transfer electrons from methyl viologen to arsenate resulting in a subsequent clearing of the gel or would alter the levels of specific activity observed *in vitro*.

These results conflict with earlier results of *arrA* expression patterns in *C. arsenatis*. *C. arsenatis* grown in the presence of arsenate and nitrate had an almost identical specific activity compared to cultures grown in arsenate alone. However, cultures grown in the absence of arsenate but the presence of nitrate, the specific activity was only 4% of the maximum (Krafft & Macy, 1998). Here, lower arsenate reductase activity is observed in cultures grown in the presence of arsenate. It should be noted, however, that *C. arsenatis* cultures began to produce more arsenate reductase with two thirds of the original specific activity after a certain time point (Krafft & Macy, 1998). This could provide insight into the lower specific activities seen here in the arsenate cultures.

For consistency between cultures, harvesting was performed at 72 hours, despite an increased growth rate in arsenate containing cultures when compared to cultures

grown in the presence of lactate alone, or lactate and either roxarsone or 2-nitrophenol. Unfortunately Krafft and Macy (1998) do not report at what phase of growth this change in specific activity occurred. If this change occurred at plateau phase, which may be suggested by their findings, then a similar change may have occurred in OhILAs cultures reaching plateau phase. This may contribute to the decreased specific activity observed here.

To reach a better understanding of *arrA* expression patterns in OhILAs, fractions grown in the presence of lactate alone, or lactate and either roxarsone, 2-nitrophenol or sodium arsenate were Western blotted using rabbit raised Anti-ArrA1 rabbit antibody which was affinity purified. Anti-ArrA1 was raised to a purified peptide strand from the *Bacillus selenitereduscens* ArrA. While there is similarity, there is only 50% identity between OhILAs ArrA and that of *B. selenitereduscens*. Low similarity, and consequently low affinity, could contribute to poor development after extended development times.

In vitro assay of roxarsone transformation

Release of OhILAs' draft annotated genome has suggested many novel enzyme targets that could be of interest to understanding the mechanism of roxarsone transformation. Among these proteins of interest, OhILAs contains a coding region specifying for a sodium dependent F_1/F_0 ATP synthase. Previous evidence has demonstrated that reduction of another nitroaromatic, caffeate, by *Acetobacter woodii* was sodium dependent (Imkamp & Müller, 2002).

The present study begins development of an *in vitro* assay aimed at detecting production of the proposed aminophenol products from transformation of roxarsone or 3-

A-4-HBAA. Here, a diazonium salt is formed between NEDD and the aminophenolic compound at low pH. Production of the amino compound can then be measured spectrophotometrically. Development and refinement of this technique is crucial to further understand the mechanism by which roxarsone is ultimately transformed into the inorganic arsenic species observed in both cultures of OhILAs as well as enrichment cultures obtained from chicken litter.

Conclusions

This present study has examined the microbial transformation of roxarsone using both a microbial physiology and proteomic approach. As previously seen, anaerobic enrichment cultures obtained from chicken litter exhibit a clostridial style transformation of roxarsone; however, cultures grown aerobically do not. Physiological studies have indicated that pure cultures of OhILAs transforming this and similar compounds containing a nitro group yield significantly higher cell yields compared to cultures grown in their absence. Taken together, these data suggest a respiratory mode of transformation.

Several key enzymes have been identified in the annotated genome of OhILAs. Among these are components of both the respiratory arsenate reductase mechanism (*arr*) and the resistance arsenate reductase mechanism (*ars*), as well as components of an anaerobic respiratory chain such as a sodium dependent F_1/F_0 ATP synthase. Despite evidence suggesting that cultures grown under varying conditions are able to reduce arsenate to arsenite, the expression pattern of ArrA1 remains unclear, as does its pattern of localization within the cell.

Future directions

This study lays crucial groundwork for the development of a working model of roxarsone transformation. The data presented here strongly support a theme where respiratory processes are coupled to initial transformation of the nitro group; however, there is still much work to be done to solidify these results. Firstly, while there is mounting evidence to suggest this is a respiratory process, the results are not yet conclusive.

It will be interesting to understand what additional compounds OhILAs is capable of transforming in a respiration-like pattern. Chemically, arsenate is analogous to phosphate, and behaves much the same way in biological systems. A radioactively labeled phosphorous analog will allow much more detailed study of the mechanism behind the transformation. Additionally, cultures grown in the presence of thiosulfate or perhaps nitrate will allow a much better understanding of the free energy requirements of the system.

Growth experiments conducted in the presence of roxarsone with OhILAs strains that have had components of the proposed respiratory chain deleted will be very informative. Further genetic techniques can also be employed to better understand the results of *in vitro* assays. It will be interesting to examine the physiology of nitroreductase negative strains of OhILAs grown in the presence of arsenate alone and arsenate and nitrate. *In vitro* nitrate reduction assays, similar to the arsenate reduction assays performed here, can be performed to determine the specific activity of arsenate reductase grown in the presence of nitrate. Additionally, *in vitro* assays performed on

OhILAs$^{\Delta arr}$ strains will be informative about the possibility of promiscuous enzymes contributing to erroneous results.

Additionally, as mentioned, initial success with *in vitro* assays to monitor disappearance of roxarsone with protein suspensions is crucial. Refinement of this technique, as well as the technique to detect concentrations of the amino-organics will be necessary. This will allow not only data to be presented on the disappearance of the initial compound, but concentrations of the final product will be quantifiable.

Beyond this, comparative studies investigating the proteomic response of OhILAs will be necessary. The annotated genome has revealed several coding regions of interest. Already, certain protein bands of interest have been identified from single dimension SDS-PAGE gels (Figure 23) and are undergoing MS/MS analysis by Peter Chovanec. Two dimensional gel electrophoresis of samples grown under the different conditions will reveal further exciting and unexpected differences between the proteomes, confirming involvement of certain enzymes, and perhaps demonstrating involvement of novel enzymes in the pathway.

There has been limited success with this technique, though the results are far from reliable. Unfortunately, the annotated genome suggests that the proteins of present interest have a high likelihood of being either integral membrane proteins or membrane associated proteins. Two-dimensional gel electrophoresis will be a powerful technique to employ, however, the difficulties associated with 2D-PAGE of membrane proteins must be overcome. CHAPS solubilization of fractions may provide initial success towards development of this technique for these purposes.

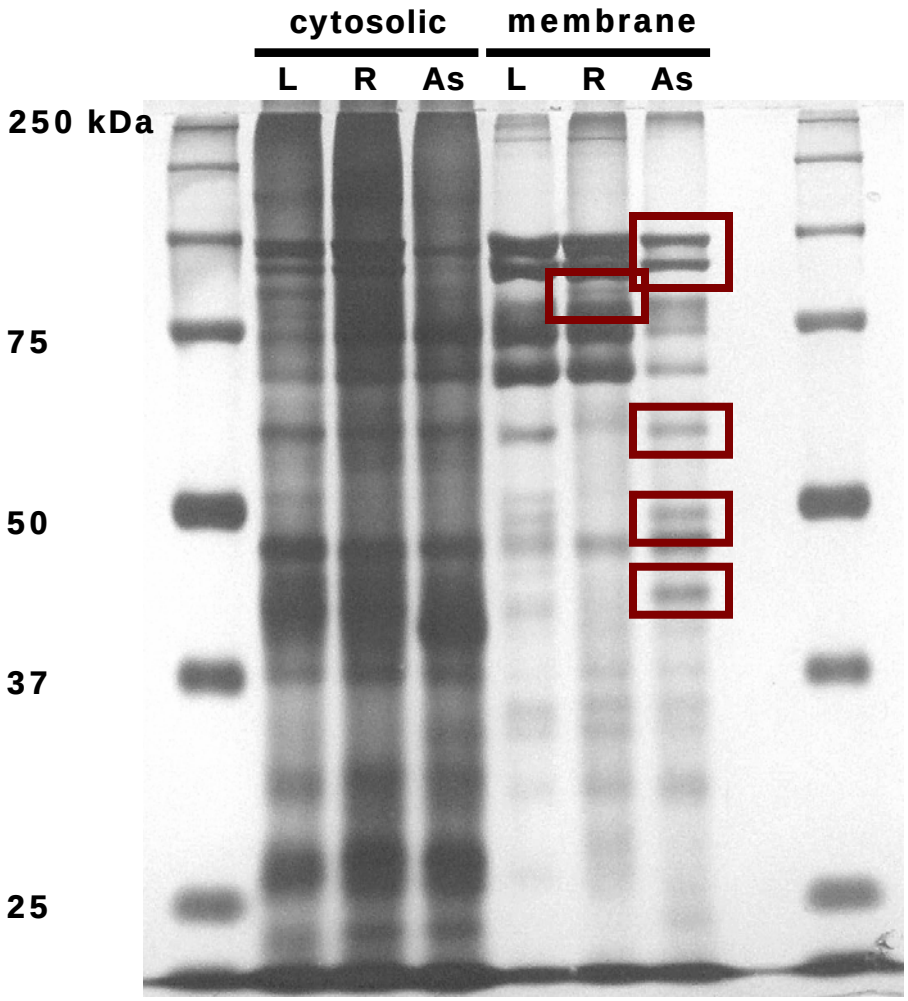


Figure 23: Single dimension SDS-PAGE of OhILAs cytosolic and membrane fraction grown in the presence of lactate alone (L) or lactate and either roxarsone (R) or arsenate (As). Bands indicated are undergoing MS/MS analysis.by Peter Chovanec.

Additionally either native purification, or purification of overexpressed enzymes in another organism, will provide additional avenues to pursue. Currently, western blot analysis is difficult because of the low homology between *Bacillus selinitereduscens* ArrA1 and that of other organisms. Purification of the OhILAs ArrA1 will allow primary antibodies with better specificity and affinity allowing better analysis of expression patterns under a variety of conditions. Purification of these enzymes will also allow more detailed analysis of enzyme activity as determined by *in vitro* assays.

Of primary interest, however, is development of a workable model for transformation of roxarsone. Results of Cortinas and colleagues (2006) have demonstrated complete transformation of the proposed metabolites from 3-nitro transformation from sewage sludge. Unfortunately, as detailed at length, this data is from a complex microbial community, the composition of which has yet to be determined. Ultimately, the key to understanding metabolism of 3-A-4-HBAA and subsequent intermediates in the process may lie in better understanding the mechanism of transformation from roxarsone to 3-A-4-HBAA. To date, evidence documenting the role environmental changes, such as changes in pH coupled to transformation of roxarsone, may have on this mechanism possibly leave an insurmountable void until this process is understood. Ultimately, a solid characterization of the mechanism and enzymes involved in this pathway is crucial, and should provide for exciting research in the ensuing years.

Appendix 1

Vitamin Mix:

1. Biotin – 2 mg/L
2. Folic Acid – 2 mg/L
3. Pyridoxine HCl – 10 mg/L
4. Riboflavin – 5 mg/L
5. Thiamin – 5 mg/L
6. Nicotinic Acid – 5 mg/L
7. Pantothenic Acid – 5 mg/L
8. p-Aminobenzoic Acid – 5 mg/L
9. Thioctic Acid – 5 mg/L
10. B_{12} – 0.1 mg/L

Filter sterilize. Store at 4^{o}C in light protective bottle.

Mineral Mix:

1. Nitrilotriacetic acid – 1.5 g/L
2. Magnesium sulfate – 3.0 g/L
3. Manganous chloride – 0.444 g/L
4. Sodium chloride – 1.0 g/L
5. Ferric chloride – 0.067 g/L
6. Calcium chloride; dihydrate – 0.1 g/L
7. Cobalt chloride – 0.1 g/L
8. Zinc sulfate 7-hydrate – 0.274 g/L
9. Cupric sulfate 5-hydrate – 0.01 g/L
10. Aluminum potassium sulfate – 0.01 g/L
11. Boric acid; granular – 0.01 g/L
12. Sodium molybdate – 0.025 g/L
13. Nickelous chloride 6-hydrate – 0.024 g/L
14. Sodium tungstate; hydrate – 0.025 g/L

Filter sterilize. Store at 4^{o}C in light protective bottle.

Phosphate Buffered Solution:

1. Sodium chloride – 8 g/L
2. Sodium phosphate (dibasic) – 0.2 g/L
3. Potassium phosphate (monobasic) – 1.15 g/L
4. Calcium chloride $CaCl_2 \cdot 2H_2O$ – 0.1 g/L
5. Magnesium chloride $MgCl \cdot 6H_2O$ – 0.15 g/L

Store at 4^oC

Results of Protein BLAST w/ ArrA Chrysiogenes arsenatis

```
Query  78   EGEWKPSGCLGCTSWCAKQVYIVDGRAIKIKANDESKIHGGNDCPRAHLAVQQVYDADRI  137
            EG+W PS C GCT+WC  +         K  A   SK + G  C R HL +QQ+YD DRI
Sbjct  42   EGKWIPSTCQGCTTWCPVEFLFRMAVRSKYAATQLSKANNGYCCVRGHLMLQQLYDPDRI  101

Query  138  KQPMKRTNPKKGRDEDPMFVPISWD-EAMDTLADKIMELRTNNETHKFMLLRGRYTNITD  196
            K PMKRTNP KGR EDP   P     +  DT+ADKIMELR NNETHK++L+RGRY++
Sbjct  102  KTPMKRTNPVKGRKEDPKICPYHMGMKQWDTIADKIMELRKNNETHKYLLMRGRYSDHNS  161

Query  197  LFYSSMPKIIGSPNNISHSSICAEAEKFGPYYTQGYWNYRDYDVQNTKYMICWGVDPLCS  256
            +FY  + K+IGSPNNISHS+ICAE EK G   T+G+W YRDYD+ N KY+I W  DPL S
Sbjct  162  IFYGDLTKMIGSPNNISHSAICAEVEKMGSMATEGFWGYRDYDLDNMKYLIAWACDPLSS  221

Query  257  NRQVSHYLNVFGELLKKGVKIVTIDPKYSSTAAKSHVWMPVIPGQDGALATAMAHVILAE  316
            NRQ+ + +     ++ +G K+V +DP+ ++TA+K+  W+P+ P +DGALA AMAHVI+ +
Sbjct  222  NRQIPNAIRKIQGVMDRG-KVVAVDPRMNNTASKAQEWLPIKPSEDGALALAMAHVIITK  280

Query  317  GLWSREFVGDFTDGNNAFISGKNVKEETFEEIHTHGVVKWWNLELKDRTPEWAEAICGVD  376
            GLWS+EFVGDF DG N F++GK VKEE FEE  T+G+VKWWNLE+KDRTP+WA  + G+D
Sbjct  281  GLWSKEFVGDFKDGKNKFVAGKTVKEEDFEEKLTNGIVKWWNLEVKDRTPKWAAKVTGID  340

Query  377  AATIRNVAIEFAKAAPYSMVFMGGGSNMQVRGAYNSMAVHALNGLVGCIDHEGGVLTGRS  436
             ATI  VA EFA+AAP   ++ G   NMQ RG+Y  M +HALNGLVG  D EGG+ TG
Sbjct  341  EATIIRVATEFAQAAPACAIWYG--PNMQPRGSYAVMCIHALNGLVGASDSEGGLCTGMG  398

Query  437  AKLNSLPGPDDFMDEMAKTNSKNKKMDQRGTKQFPALKEGKPGSGVITNRVADAILAEDP  496
            +  +S P  D + D++AK  +KNKK+DQRGT +FPA+   KPG+GV+TN VADA+LA DP
Sbjct  399  SPSSSYPKIDAYQDDVAKAGAKNKKIDQRGTLKFPAMGSAKPGTGVVTNNVADALLAADP  458

Query  497  YLPKVVIGYFCNFNYSCPGTDRWDKAMSKIEFFAHCVTHYSEMSHFADLLLPSTHHMFEQ  556
            Y  KV IGYFCNFN+S     RWDKA++K+ FF HCV  +SEM++FAD++LP+  H  E
Sbjct  459  YDIKVAIGYFCNFNFSGTDGARWDKALAKVPFFVHCVPMFSEMTYFADIVLPAALHHTED  518

Query  557  MASAAQKANSYTHLWITQRLIEPVYDVKNPESEVLWMLAEKLEQRGFSNLLDFC-KTVKD  615
             A    KAN + H  I Q ++E ++DVK  E+E+ W+LAEKL+ +GF N+ ++     KD
Sbjct  519  WAVIRSKANLHGHTSIQQPVVERMFDVKGVETEITWLLAEKLKAKGFENMYNWLYNEYKD  578

Query  616  PETGSEPTNGLEFELYAYKTLTQSIWDPTKYTELENHGDKFNSWNEFIQKGVWNSDPYKF  675
            PETG  PTN LEF LYA K  ++  WDP +  E +  GDK N W +F++KG+ NS  +KF
Sbjct  579  PETGKNPTNSLEFALYATKIRSKKCWDPKENAEYK--GDKLNGWADFMEKGIVNSPKFKF  636

Query  676  KGLWSK-MKTETTKFEFYSETLKKALQEHADKHNTDVDDILATCKYTGKGEQAFIPHYEP  734
            +  W K   TET KFEFYSETLKK L  HA+K+   VD ++    Y  +GE AFIPHYE
Sbjct  637  RQKWEKGFPTETKKFEFYSETLKKGLLAHAEKNKVTVDQVMEATNYEARGELAFIPHYES  696

Query  735  PYMEGKVEQYPFAYIDSKSRLAREGRSANCSWFQEFKDADPGDIKWSDCIKMNPKDAIEL  794
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Sbjct  697  PKRHGDVKEFPFSLIDMKSRLNREGRSTNATWYHAFKKCDPGDVNQEDVLQINPADAKKL  756

Query  795  GLKDGDKVKVTSPTGEIVTTLKLWEGLRPGTVQKNYGQGHWAMGHVAAEDFEKKLSRGGN  854
            G+ +GD VKVTS  G +    +LWEG+RPG V K YGQGH+AMG V+A+DF K ++RG N
Sbjct  757  GINEGDMVKVTSVIGSLTVKARLWEGVRPGCVAKCYGQGHFAMGRVSAKDFGKAVARGAN  816

Query  855  SNVIIPADYERLSGATAYYGSFR-VKIERA  883
             N I+PADY+R++GATA  G F  VKIE+A
Sbjct  817  FNDIMPADYDRITGATARNGGFTGVKIEKA  846
```

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