ABSTRACT

Title of Document: A STUDY OF UNUSUAL METABOLIC VARIANTS OF AEROMONAS CAVIAE AND AEROMONAS HYDROPHILA USING A POLYPHASIC TAXONOMIC APPROACH

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Variation in acid production from carbohydrate metabolism has been identified in *Aeromonas* as a potential indicator for new subspecies. Therefore, pure cultures of non-lactose fermenting *Aeromonas caviae*, a cause of waterborne infections in humans and other vertebrates, were studied after noting a mixture of acid producing and non-acid producing colonies after four days of incubation on MacConkey agar at ambient temperature. Unusual arabinose negative strains of *A. hydrophila* (usually arabinose positive) were added to the project to further study the correlation between carbohydrate fermentation and taxonomy. These metabolic variants of *A. caviae* and *A. hydrophila* were studied for phenotypic differences via carbohydrate utilization assays as well as genotypic differences via FAFLP. The results suggest that the *A. caviae* isolates MB3 and MB7 should be considered novel subspecies, while the arabinose negative strain designated *A. hydrophila* subsp. *dhakensis* is correctly identified as a subspecies of *A. hydrophila*.

A STUDY OF UNUSUAL METABOLIC VARIANTS OF *AEROMONAS CAVIAE* AND *AEROMONAS HYDROPHILA* USING A POLYPHASIC TAXONOMIC APPROACH

By

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DEDICATION

I dedicate this thesis to my parents, Chao-hsi and Hui-yen, and my sister, Zanetta. I would not have made it this far without all of their support and encouragement.

I would also like to dedicate this project to Dr. Sam W. Joseph, who taught me all about the world of scientific research; without his infinite knowledge and patience I would not have been able to finish this degree.

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INTRODUCTION

Aeromonas Physiology and Biochemical Profile

As described in the most recent edition of Bergey's Manual of Systematic Bacteriology, aeromonads are gram-negative, oxidase-positive, glucose-fermenting, facultatively anaerobic rods belonging to the family *Aeromonadaceae* (Martin-Carnahan and Joseph, 2005). As most *Aeromonas* species are mesophilic and motile, these organisms are ubiquitous in fresh, brackish, chlorinated, and non-chlorinated water and have been isolated from estuarine and marine water sources around the world. Aeromonads are more numerous during the warmer parts of the year and can be found in a variety of sources ranging from biofilms, biosolids, and sewage, to raw meat, fish, seafood, and vegetables (Hazen et al. 1978; Seidler et al. 1980; van der Kooj, 1988; Kaper et al. 1981; Holmes and Niccolls, 1995; Holmes et al., 1996).

Aeromonas Virulence and Pathology

Aeromonas species possess a diverse array of virulence factors: various endotoxins, hemolysins, enterotoxins, and adherence factors have been identified, though the precise role of each factor in pathogenesis is currently unknown. Aeromonads use these virulence factors to cause either primary or opportunistic disease in a variety of vertebrate and invertebrate hosts. Due to the abundance of

Aeromonas in aquatic environments, it is not surprising that *Aeromonas* spp. has been isolated from aquatic organisms including frogs, freshwater and saltwater fish, and even leeches (Sanarelli, 1891; Caselitz, 1955; Gosling, 1996a; Austin and Adams, 1996; Graf, 1999a; Huys et al. 2003, Martinez-Murcia et al. 2008). In fish and frogs, this microbe has been shown to cause the formation of lesions and cellulitis (Joseph and Carnahan, 1994; Huys et al. 2003; Martinez-Murcia et al. 2008).

The human pathology associated with *Aeromonas* falls largely into two groups: gastroenteritis and wound infections, both of which can occur with or without bacteremia. In healthy humans, especially children, gastroenteritis is the most common manifestation, usually following the ingestion of contaminated water or food. However, *Aeromonas* also has the capability to cause wound infections (following exposure to contaminated water) and more serious systemic disease (peritonitis, sepsis, or meningitis). Sepsis, peritonitis, and meningitis generally arise secondary to other conditions but are associated with high mortality rates; mortality is especially high in patients with liver cirrhosis, underlying disease, or in patients who are immunocompromised (Janda and Abbott, 1996; Janda and Abbott, 1998; Janda and Abbott, 2010; Altwegg 1999). *Aeromonas* spp. were also isolated from wounds of tsunami victims in Thailand (Hiransuthikul et al. 2005) and species capable of causing necrotizing fasciitis were found in floodwater samples during Hurricane Katrina in New Orleans (Presley et al. 2006).

Taxonomy Overview

Historically, *Aeromonas* taxonomy was very convoluted, but over the past twenty years it has been much more clearly elucidated. Currently, there are approximately eighteen species as defined at the phenospecies level, compared to approximately seventeen hybridization groups (HGs), or genomospecies, when classified by DNA homology (Martinez-Murcia et al. 2008). Most phenospecies of *Aeromonas* are associated to some extent with human disease; however, the most important human pathogens are *A. hydrophila*, *A. caviae*, and *A. veronii* biovar sobria.

The various aeromonads fall into two major groups: motile, mesophilic organisms first described broadly as *A. hydrophila,* and non-motile, psychrophilic aeromonads originally classified as *A. salmonicida.* While non-motile aeromonads present with characteristics infrequently observed in other *Aeromonas* species, DNA hybridization studies indicated that these organisms are still closely related to the motile aeromonads (MacInnes et al., 1979). This observation suggests that organisms belonging to the genus *Aeromonas* are derived from two major evolutionary branches: a relatively homogenous group of non-motile organisms belonging to *A. salmonicida*, and a diverse group of motile aeromonads encompassed by the remaining *Aeromonas* species.

Importance of Proper Taxonomic Designation in Epidemiology and Research

The importance of identifying organisms to the species and sub-species level when tracking organisms in disease outbreaks cannot be understated. In the clinical setting, accurate identification of an isolate through the use of proper taxonomic designation allows for better diagnosis and increased efficacy during treatment. Likewise, in research, it is highly important to identify the exact organism of interest before investing a large amount of time and resources into a project. Therefore, developing precise taxonomical systems and identifying new species and subspecies serve not only to satisfy scientific curiosity, but also to improve medical treatment and the efficiency of future research. This aspect of taxonomy has become so important that an increasing variety of molecular, serologic, phage typing and other methods have been implemented in order to classify various species of bacteria. Recent cases in point include *Salmonella* isolation in foodborne outbreaks along with recognition of *Yersinia enterocolitica*, *Aeromonas*, and *Staphylococccus aureus* subtypes (Peterson et al. 2010; Neubauer et al. 2001; Huys et al. 2002; Martinez-Murcia et al. 2008; Hirose et al. 2010).

Historical Taxonomy

The first description of a motile aeromonad was reported by Sanarelli in 1891, when he isolated a bacterium from an infected frog which he termed *Bacillus*

hydrophilus fuscus (Sanarelli, 1891). Sanarelli was able to induce septicemia by reintroducing the isolated bacillus into a variety of cold and warm-blooded animals, demonstrating this organism's capability to cause disease. The tripartite taxonomic designation *Bacillus hydrophilus fuscus* remained in use until 1901, when the organism was renamed *Bacterium hydrophilum* by Chester (Chester, 1901). Over the following sixty years, similar organisms were isolated from a variety of animals including fish, frogs, snakes, livestock, and birds (Gosling, 1996a); however, they were classified as members of other genera, including *Achromobacter, Aerobacter, Escherichia, Flavobacterium, Proteus, Pseudomonas,* and *Vibrio*. These classifications proved to be inadequate, resulting in Kluyver and Van Niel proposing the new genus *Aeromonas* ("gas-producing unit") in 1936. Their classification was validated by taxonomic studies seven years later (Stanier, 1943) and led to recognition of the genus in Bergey's Manual of Determinative Bacteriology in 1957 (Snieszko, 1957).

Non-motile aeromonads were first isolated by Emmerich and Weibel in 1894 from trout (Emmerich and Weibel, 1894); and further study of similar bacteria that caused furunculosis in fish resulted in the species *Bacterium salmonicida* (Lehmann and Neumann, 1896). These non-motile organisms were discovered to be psychrophilic, with ideal growth temperatures of 10-15^{o}C, in contrast to the majority of aeromonads. They are studied to this day due to their economic implications for

aquaculture (Austin and Adams, 1996; Hänninen et al. 1995; Hänninen et al. 1997). Following the establishment of the genus *Aeromonas* and additional taxonomic studies conducted at the Centers of Disease Control (CDC), three species were suggested: *A. hydrophila, A.shigelloides* (now its own genus, *Plesiomonas*),and the designation *A. salmonicida* (for the nonmotile, pyschrophilic organism first described as *Bacterium salmonicida)*.

Taxonomy Reform

The next major revision in *Aeromonas* taxonomy occurred when Popoff and Veron used numerical taxonomy, as defined by Sneath and Sokal (Sneath and Sokal, 1973), to analyze 68 motile, mesophilic *Aeromonas* isolates for 203 morphological, biochemical, and physiological characteristics (Popoff and Veron, 1976). Numerical taxonomy allowed Popoff and Veron to divide the isolates taxonomically on the basis of 50 specific phenotypic characteristics into two distinct categories: *Aeromonas hydrophila* (biovar X_1 and X_2) and a new species, *Aeromonas sobria* (biovar Y). Popoff et al. later expanded their preliminary numerical taxonomy study by analyzing 55 motile, mesophilic aeromonads genetically via S1 nuclease DNA/DNA hybridization (Popoff et al., 1981). This lead to the establishment of another new species: *Aeromonas caviae* (formerly biovar X_2), and the refinement of *Aeromonas hydrophila* to include only biovar X_1.

While DNA/DNA hybridization allowed for greater resolution than biochemical testing, Popoff et al. noted the limitations of the technique, as each of the three species described contained at least two or three distinct hybridization groups that shared phenotypic traits. Strains classified as belonging to DNA HGs (or genomospecies) 1-3 were all *A. hydrophila*; DNA HGs 4-6 comprised phenospecies *A. caviae*; and *A. sobria* represented DNA HGs 7-8. Each of these three phenospecies was included, along with a description of the non-motile species *A. salmonicida,* in the description of *Aeromonas* under the family *Vibrionaceae* for the First Edition of Bergey's Manual of Systematic Bacteriology in 1984 (Popoff, 1984). Work later performed at the CDC generated additional DNA/DNA hybridization data by utilizing the hydroxyapatite method (rather than the S1 nuclease method used by Popoff et al.) of DNA/DNA hybridization at 60^{o}C; a varied number of clinical, environmental, and reference strains of *Aeromonas* were analyzed and organized into 12 different DNA hybridization groups (Fanning et al. 1985; Farmer et al. 1986).

At that time, the authors recommended using the terms "*A. hydrophila* complex", "*A. sobria* complex", etc. when referring to aeromonads, thus causing the genus to be indeed "complicated". In 1979, Joseph et al. published the first paper reporting on *A. sobria* as a human pathogen; the organism was isolated from a soft tissue infection in the leg of a Navy diver (Joseph et al. 1979). This finding served as a catalyst for Joseph and Carnahan to move toward more specific taxonomic

identification of the aeromonads. Ironically, this organism was later found by Carnahan and Joseph, using more refined identification methods, to be *A. jandaei* (Carnahan et al. 1991).

A further major development occurred in 1986 when it was demonstrated that aeromonads were evolutionarily divergent and equidistant from *Enterobacteriaceae* and *Vibrionaceae* (Colwell et al. 1986). Through the use of 16S rRNA cataloguing, 5S rRNA sequence comparison, and rRNA/DNA hybridization data, Colwell et al. proposed that the genus *Aeromonas* should belong in its own family, *Aeromonadaceae*, rather than *Vibrionaceae*. This suggestion was later supported by additional 16S rDNA sequencing of reference strains, which indicated that aeromonads formed their own distinct branch in a phylogenetic tree under the gamma subclass of *Proteobacteria* (Martinez-Murcia et al. 1992a).

Taxonomy Refinement

In the late 1980's and early 1990's, several broad taxonomic studies were performed by Kuijper et al., Altwegg et al., and Carnahan et al. in the hopes of clarifying *Aeromonas* taxonomy. Due to different classification systems, there were eight proposed phenospecies at that time, as opposed to thirteen known genomospecies (hybridization groups). Kuijper et al. used an array of 30 phenotypic traits to analyze a collection of 189 human fecal samples, where it was discovered

that the majority of isolates were phenotypically classified as *A. hydrophila, A. caviae,* and *A. sobria* (Kuijper et al. 1989). When these strains were further subjected to DNA/DNA hybridization, however, it was found that they primarily belonged, as predicted by previous work, to DNA hybridization groups 1, 4, and 8, with an increased frequency of the rarer hybridization groups (2, 3, 5A).

The next study was conducted, with assistance from the CDC, by Altwegg et al. in 1990. The culture collection consisted mainly of fecal isolates from Swiss patients, with a handful of environmental strains from Germany. In addition to the tests used by Kuijper et al., Altwegg et al. analyzed a total of 63 different phenotypic characteristics by utilizing API-20E, API-50E, and API ATB 32GN identification strips (API Systems, La Balme-Les Grottes, France). S1 nuclease DNA hybridization results were scored for similarity by computer analysis and clustered using the **U**nweighted **P**air **G**roup **M**ethod with arithmetic **A**verages (UPGMA) previously used by Popoff and Veron (Sneath and Sokal, 1973; Popoff and Veron, 1976). By using a cluster analysis cutoff level of 88% similarity, three major clusters, or phenons, were described that matched three species (*A. hydrophila, A. caviae,* and *A. sobria*) previously described by Popoff in Bergey's Manual of Systematic Bacteriology; however, each phenon contained more than just one genomospecies or phenospecies.

Altwegg et al. extended their study by further studying the strains through DNA/DNA hybridization and made several interesting observations. As also determined by Kuijper et al., the majority of the sampled strains were found to belong to DNA HGs 1, 4, and 8, while many of the remaining strains belonged to HGs 2, 3, and 5. Clinical isolates tended to belong to HGs 1, 4, 5A, 5B, and 8 while environmental strains were located mainly in HGs 2, 3, and 5A, suggesting that certain hybridization groups possessed greater human virulence potential.

More importantly, all but one of the clinical strains resembled *A. sobria* (originally described as HG 7) phenotypically but belonged to DNA HG 8 instead. HG 8 was of interest due to its genetic similarity to HG 10, the newly described ornithine-positive *A. veronii* (Hickman-Brenner et al. 1987). While HG 7 and HG 8 were different genetically but very similar phenotypically, HG 8 and HG 10 were very similar genetically and remarkably different phenotypically, especially when arginine dihydrolase activity, ornithine decarboxylase activity, and esculin hydrolysis were compared.

In their paper establishing *A. veronii*, Hickman-Brenner et al. did suggest that HG 8 and 10 might in fact be biogroups of *A. veronii* due to their genetic similarity. This hypothesis was later supported by Altwegg et al., who published additional results to propose that DNA HG 8Y (which included the clinical *A. sobria* isolates) and

DNA HG 10 (*A. veronii*) should be considered as biotypes or subspecies of *A. veronii* despite their biochemical differences (Altwegg et al. 1990). These proposals clearly suggest that biochemical differences between two groups of strains can be used as the basis for defining new subspecies or biotypes. Joseph et al. later proposed in a case study that HG 10 (ornithine positive *A. veronii*) and HG 8 (ornithine negative *A. sobria*) should be considered *A. veronii* biovar veronii and *A. veronii* biovar sobria, respectively (Joseph et al. 1991). This paper suggested that all previously described *A. sobria* clinical isolates are in fact *A. veronii* biovar sobria, and not the environmentally isolated *A. sobria* (HG 7) first proposed by Popoff and Veron.

In 1993, Carnahan and Joseph published the next significant *Aeromonas* taxonomic study. The examination of 167 motile aeromonads from diverse geographical locations (Bangladesh, Egypt, India, Indonesia, Somalia, Sudan, and the US) distinguished this study from the studies mentioned above, where isolates were taken from one major geographical source (such as the Netherlands or Switzerland). Additionally, a wide variety of clinical, non-fecal samples were included in the study in the hopes of avoiding any species detection bias due to clinical isolation methods or geographic location (Carnahan and Joseph, 1993).

Carnahan and Joseph initially examined the 167 strains on the basis of 80 phenotypic traits in order to perform numerical taxonomic analysis using the

SAS/TAXAN^{R} program and clustering by the UPGMA technique. With an 85% similarity coefficient as the species designation cutoff, the strains were grouped into 12 phenons, with two small clusters of atypical strains. A large cluster of HG 8 strains was shown to merge at a similarity value of 84% to a cluster of HG 10 *A. veronii,* reinforcing the suggestion by Joseph et al. that the two biochemically distinct groups were in fact biovars of the same species. Further analysis of the numerical taxonomy via dendrogram analysis revealed that HGs 2, 3, and 7 were primarily environmental or veterinary strains, while HGs 9, 10, 12 were exclusively extraintestinal clinical isolates. Later analysis of HG 2 resulted in the proposal of a new species, *A. bestiarum* (Ali et al. 1996).

One of the two small clusters of atypical strains was shown to merge to the DNA definition strain for HG 9 with a similarity of 84%. When this hybridization group was first established in the early 1980's, there were too few isolates belonging to the group for it to be considered as a new species. By grouping the atypical strains with similar strains from other collections and performing additional genotypic and phenotypic testing, Carnahan and Joseph were able to propose the new species *A. jandaei*, on the basis of negative reactions for sucrose fermentation and esculin hydrolysis (Carnahan et al. 1991). Similarly, the other atypical cluster noted in the Carnahan and Joseph 1993 study were all isolated from the same general locale in Southeast Asia. After evaluation and combining them with other

strains from Indonesia, phenotyping and DNA/DNA hybridization showed that this cluster represented another new species, *A. trota*: negative for esculin hydrolysis, Voges-Proskauer, and susceptible to ampicillin, a rare trait among aeromonads (Carnahan et al. 1991b).

The studies performed in the 1980's and early 1990's established the success of a polyphasic approach combining genomic and phenotypic analysis for the establishment of new Aeromonas species. In the past two decades, a variety of additional species have been described using this approach, including *A. eucrenophila, A. schubertii, A. allosccharophila* (Martinez-Murcia et al. 1992b), *A. encheleia* (Esteve et al. 1995), and *A. popoffi* (Huys et al., 1997), with three additional species – *A. simiae* (Harf-Monteil et al. 2004), *A. molluscorum* (Miñana-Galbis et al. 2004), and *A. bivalvium* (Miñana-Galbis et al. 2007) bringing the total to seventeen phenospecies. *A. aquariorum* (Martinez-Murcia et al. 2008) is currently being examined as a potential new species of *Aeromonas*, resulting in a total of eighteen phenospecies -- a far cry from the 4 first described by Popoff and Veron in 1984 for the First Edition of Bergey's Manual of Systematic Bacteriology.

Molecular Identification Methods

The use of DNA/DNA hybridization emerged in the 1980's as the preferred standard for species classification and played a major role in clarifying *Aeromonas* taxonomy (Popoff et al. 1981). While the technique allowed for finer distinctions than biochemical testing, DNA/DNA hybridization was a complex technique that required stringent laboratory conditions and extensive training. Due to issues with the repeatability and complexity of the protocol, other techniques and approaches have been sought.

Within the last two decades, **F**luorescent **A**mplified **F**ragment **L**ength **P**olymorphism (FAFLP) analysis has emerged as a premier diagnostic tool for *Aeromonas* taxonomy (Janssen et al. 1996; Huys et al. 1996). Previous efforts had been focused on other methods such as metabolic differences (sugar utilization, breakdown of urea), DNA hybridization, or pulsed-field gel electrophoresis; however, FAFLP has been shown to allow high throughput with excellent resolution and reproducibility. When combined with metabolic assays as part of a polyphasic approach, FAFLP offers a flexible, accurate, and sensitive method for the classification and identification of new species.

Aeromonas hydrophila

A. hydrophila, the first motile aeromonad to be reported (1891), remains an organism of interest to this day. *A. hydrophila* (DNA HG 1, 2, and 3) can be isolated from fresh and marine waters, and is able to induce septicemia when introduced into a variety of cold and warm-blooded animals; it is clinically important as a major cause of both intestinal and nonintestinal disease in humans. *A. hydrophila* remains closely associated with gastrointestinal disease, due to its expression of cytopathic and cytotoxic enterotoxins, such as β-hemolysin (Houston et al. 1991; Cahill 1990; Janda 1991; Thornley et al. 1997). *A. hydrophila* can also be linked to hemolytic uremic syndrome (HUS) (Bogdanovic et al. 1991), septicemia (Janda et al. 1994), peritonitis (Munoz et al. 1994), and meningitis (Parras et al. 1993).

Aeromonas caviae

Aeromonas caviae was first described by Popoff et al. in 1981 following DNA/DNA hybridization analysis of 55 motile *Aeromonas* strains (Popoff et al. 1981). This species was formerly considered the "anaerogenic" biovar (biovar X_2) of *A. hydrophila* due to the lack of gas production from glucose and the absence of H_2S when grown on GCF medium; additionally, *A. caviae* could be distinguished from *A. hydrophila* by negative reactions for elastase production and Voges-Proskauer. The phenospecies designation *A. caviae* encompasses organisms belonging to

hybridization groups 4, 5A, and 5B; some environmental isolates produce acid from lactose, while others do not.

A. caviae can commonly be found in fresh water or sewage; it has also been isolated from a variety of animals, birds, and fish. *A. caviae* mainly causes gastroenteritis, although specific methods of pathogenicity are relatively unknown. It has been shown, however, to cause alterations and enteropathogenic effects in mouse small intestine (Longa-Briceño et. al 2006) and has been implicated specifically as the leading cause of *Aeromonas*-related diarrheal disease in pediatric patients (Altwegg and Johl 1987). In humans, *A. caviae* can also cause septicemia (Janda et al. 1994) and other extra-intestinal disease, but does so primarily in immunocompromised humans (Janda and Abbott 1998).

Scope of Project

Aeromonas caviae

The *A. caviae* isolates studied in this project were cultured by Dr. Amy Horneman from pure non-lactose fermenting stocks that were streaked on MacConkey agar (MAC), incubated at 37^{o}C overnight (O/N), and were left on the lab bench for approximately 4 days at ambient temperature. At the end of this period, it was discovered that each plate contained several distinct variants: acid producing (AP), non-acid producing (NAP), and weak acid producing (WAP) colonies, even

though the original cultures were non-acid producing. Further characterization and subculturing showed that these phenotypes were persistent, suggesting the presence of new subsets of *A. caviae*.

Aeromonas hydrophila subsp. *dhakensis*

In 2002, Huys et al. described a new arabinose negative subspecies of *A. hydrophila*, *A. hydrophila* subsp. *dhakensis* (Huys et al. 2002) isolated from Bangladeshi children. By utilizing a polyphasic approach emphasizing FAFLP, ERIC-PCR, DNA-DNA hybridization and biochemical testing, it was suggested that the genetic and metabolic idiosyncrasies of the isolates did not digress enough for the isolate to be considered a new species, but rather, a subspecies. FAFLP testing showed a 36% similarity in peak profiles between "*dhakensis*" and arabinose positive HG 1 A. *hydrophila*; FAFLP requires <35% similarity (greater than 65% difference in FAFLP profiles) in order to be classified as a completely new species. Additionally, DNA-DNA hybridization showed 78-92% hybridization between "*dhakensis*" and other HG 1 *A. hydrophila*, a value significantly higher than the traditional 70% cutoff.

Previously, while working in Dr. Joseph's laboratory, Dr. Horneman discovered a small group of *A. hydrophila* which were non-acid producers on arabinose. After establishing a dendrogram of all of her *Aeromonas* isolates, a group of arabinose negative strains (cluster I) were grouped immediately adjacent to the

arabinose positive strains (cluster H). Other phenotypic differences between the clusters included greater frequency of acid produced from salicin by cluster I aeromonads, and greater resistance to ceftazidime (95% to 65%), imipenem (60% to 0%), and cefoxitin (95% to 0%).

Recently, some dispute has arisen after Martinez-Murcia et al. suggested that the *A. hydrophila* subsp. *dhakensis* strains actually belonged to the recently defined species *A. aquariorum* (Martinez-Murcia et al. 2008) due to metabolic similarities and MLST (**M**ulti-**L**ocus **S**equence **T**yping) patterns. Martinez-Murcia suggested that Huys' metabolic profile of "*dhakensis*", especially utilization of lactose, L-fucose, and urocanic acid, matched the description of *A. aquariorum*. Additionally, MLST analysis of the "*dhakensis*" strains showed significant differences from HG 1 *A. hydrophila with* 15-23 differences in concatenated housekeeping genes; therefore, Martinez-Murcia hypothesized that the "*dhakensis*" strains did not belong to *A. hydrophila* but rather the new species *A. aquariorum*. While this debate is still ongoing, it indicates once again that differences in sugar utilization combined with genetic analysis (FAFLP or MLST) can allow one to identify and classify new species or even subspecies of *Aeromonas*.

Hypothesis

With the recent success of using FAFLP in conjunction with metabolic assays for the identification of the subspecies *Aeromonas hydrophila* subsp*. dhakensis* and *Aeromonas hydrophila* subsp. ranae (Huys et al. 2002; Huys et al. 2003), it is proposed that applying these same techniques will identify the *A. caviae* metabolic lactose variants as new subspecies. Furthermore, by studying a large group of well characterized arabinose negative *A. hydrophila* that are phenotypically suspect *A. hydrophila* subsp. *dhakensis*/*A. aquariorum* using FAFLP, the classification of *A. hydrophila* subsp. *dhakensis* as a separate subspecies of *A. hydrophila* can either be confirmed or denied.

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MATERIALS AND METHODS

Aeromonas Strains

Aeromonas strains (including arabinose negative *A. hydrophila*) used for reference FAFLP banding patterns (Table I)*, A. caviae* metabolic isolates (Table II), and the *A. hydrophila* subsp. *dhakensis* strain were taken from –80^{o}C freezer stock within the Joseph lab. Strain origins are further detailed in the tables mentioned above; the various metabolic mutants of *A. caviae* were first isolated by Dr. Mark Borchardt in Marshfield, WI from fecal isolates. *A. hydrophila* subsp. *dhakensis* was provided by Dr. Geert Huys through Dr. Horneman.

Media and Growth Conditions

Brain heart infusion (BHI) broth and agar (Difco) was used as the preferential non-differential growth medium, while MacConkey agar (MAC) (Difco) was used to characterize the metabolic activity of the variants on lactose. Solid media was prepared using 20 mL aliquots per plate.

Organisms were preserved for long-term storage by freezing in BHI with 20% glycerol at –80^{o}C in 3.0 mL freezer vials. Cultures revived from freezer stocks were first streaked on BHI agar and incubated overnight prior to inclusion in experimental

procedures.

Unless stated otherwise, all liquid cultures were prepared in 5 mL of broth with an overnight incubation (18-24 h) at 37^{O}C with aeration in a Series 25 Incubator Shaker (New Brunswick Scientific Company, Inc.). Solid media (agar) culture incubations at 37^{O}C under ambient atmospheric conditions were performed in a VIP Imperial II Dual Chamber Incubator (Labline Instruments, Inc.). Carbohydrate utilization medium is described in a following section.

Strain Identification

Strains for *A. hydrophila* analysis

Reference *Aeromonas* strains for FAFLP banding patterns were previously identified and characterized by Dr. Horneman (Carnahan and Joseph, 1993) using over 50 different metabolic and genetic methods (including DNA-DNA hybridization). Strains were chosen from both closely related DNA-DNA hybridization groups (genomospecies) and groups further away in order to provide a variety of reference points; when possible, strains from both environmental and clinical sources were included from within each hybridization group. Multiple *A. hydrophila* and *A. caviae* strains were included in order to provide increased resolution within the species (Table I).

A. hydrophila subsp. *dhakensis* was isolated and described in Huys et al. 2002 via metabolic testing, FAFLP, ERIC-PCR, and microplate DNA-DNA hybridization.

A. caviae metabolic variants

Seven MacConkey plates containing known *A. caviae* isolates (MB3, MB7, MB8, MB10, MB11, MB16 β-hemolysis, MB16 γ-hemolysis) with colonies presenting varying metabolic characteristics (Table II) were given to the lab by Dr. Horneman for analysis. Single acid producing (AP), non-acid producing (NAP), and weakly acid producing (WAP; WAP variants were not recovered from all strains) colonies were selected from each plate and subcultured four separate, successive times on MAC. Plates were initially allowed to grow overnight at 37^{o}C, results were recorded, and the plates were subsequently placed at room temperature for further observation (up to 4 days). Colonies with varying reactions to lactose were isolated, purified, and transferred to separate freezer vials.

Invasion Assay (*A. caviae*)

The T84 human colon cancer tissue cell line used for this assay was kindly donated by Dr. Daniel Stein. T84 cells were incubated in F12 – Dulbecco's Modified Eagles medium (DMEM media) (American Type Culture Collection) with 70 mL/L fetal bovine serum added to enrich the media and 10 mL/L antibiotic solution (100

units/mL penicillin and 100 µg/mL streptomycin) (Sigma) added to prevent unwanted bacterial growth.

One week prior to the experiment, 2.0 x 10^5 T84 tissue cells were seeded per well on a 6-well tissue cell culture plate (Corning) containing 3 mL of DMEM Media (containing penicillin and streptomycin) per well and cultured at 37^{o}C with 5% CO_2 in a humid Napco 6300 CO_2 incubator. *A. caviae* strains used in the invasion assay were revitalized from freezer stock two days in advance, incubated on BHI plates overnight, and subcultured once one day prior to the experiment. Strains used in this assay included MB3 AP, MB3 NAP, MB10 AP, and MB10 NAP.

On the day of the invasion experiment, tissue cells were washed 5 times with 2 mL of Invasion Media (DMEM with FBS, but containing no antibiotics). Bacteria were re-suspended from the overnight subculture plates into 4 mL aliquots of 0.1% saline and adjusted to provide a final Klett reading of 100 (approximately 1.0X10^9 CFU/ml). One mL aliquots of the bacterial suspensions were removed and centrifuged for 1 min at 12,000 rpm using a tabletop Spectrafuge (Labnet). The supernatant was then aspirated using a sterile Pasteur pipet, and each sample was re-suspended in 1 mL of Invasion Media. Cell suspensions were then diluted tenfold with Invasion Media to an approximate concentration of 1X10^8 CFU/mL. To each invasion well, 1 mL of the appropriate suspension was added, resulting in a 100:1

multiplicity of infection (MOI) with 100 bacteria per 1 T84 cell. The 6- well plate was incubated at 37^{o}C with 5% CO_2 for 3 h.

After 3 h, the media in all wells were aspirated. One mL of 200 ug/mL gentamicin (Sigma) in DMEM culture media and 2 mL of Invasion Media were then added to the invasion wells, and 3 mL of Invasion Media were added to the cell associated wells. The plates were then incubated for 1 h at 37^{o}C with 5% CO_2. Following the gentamicin treatment, the wells were washed 5 times with 1x PBS, and 1 ml of 0.1% Triton-X (Sigma) was then added to each well to lyse the tissue cells. The wells were incubated again at 37^{o}C with 5% CO_2 for 5 min before the cell lysates were collected in a glass test tube using a cell scraper (Sarstedt) and pipet. Each lysate solution was then pipetted up and down in the tube 20 times to break up any clumps before the re-suspended cells were serially diluted (using the dilution scheme: lysate, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}) and dilutions 10^{-3}, 10^{-4}, and 10^{-5} were plated onto BHI agar. Plates were incubated at 37^{o}C overnight and colony counts were obtained the following day. (See appendix A for detailed procedure.)

Agarose Gel Electrophoresis (*A. hydrophila* and *A. caviae*)

Agarose gel electrophoresis was used at various stages to confirm the effectiveness of DNA sample manipulation. Gels were made using 0.75% agarose with 1x TAE buffer and run at 100V. The ladder used for all gels was HyperLadder I

(Bioline) with a separation range of 200 – 10000 bp. Gels were stained for 20 minutes in 1x SYBR Green (BioRad) before visualization using a BioRad Gel Documentation System.

Polyphasic Phenotyping

ONPG (*A. caviae*)

In order to further characterize the *A. caviae* weak acid producing and non-acid producing metabolic variants, an OPNG disk assay (BD) was performed. Prior to the experiment, isolates were cultured overnight on TSI slants. An ONPG disk was placed into a sterile tube for each sample, and 0.5 mL sterile saline was added. Each tube was heavily inoculated with the test isolate and incubated at 35^{o}C for 5 h. The presence of yellow product indicated a positive test, while samples that remained clear were incubated overnight for further observation. (See appendix B for detailed procedure).

Carbohydrate Utilization (*A. hydrophila* and *A. caviae*)

Carbohydrate utilization media consisted of the following per one liter: 15 g agar (Difco), 2 g of the appropriate carbohydrate carbon source (L-arabinose, L-fucose, or lactose), 6.1 g Tris-HCl pH 7.5 (50mM), 1 g NH_4Cl, 75 mg K_2HPO_4, 28 mg $FeSO_4$-$7H_2O$, and ultra-pure water (Milli-Q) to 1L. Plates were prepared using 20 mL of media per plate. Strains were revived from freezer stock onto BHI agar one day

prior and the resulting culture was used to inoculate each of the three sugar utilization plates. Plates were incubated overnight at 37°C and checked for growth; the ability to grow on the agar signified the ability to utilize the sugar. Due to low initial growth, plates were incubated for a second 24h before each sample was subcultured onto a fresh sugar utilization plate and observed once again. If no growth was observed, the plate was placed back in the 37°C incubator for up to 4 days for further observation. (See appendix C for detailed procedure.)

Fluorescent Amplified Fragment Length Polymorphism (FAFLP) analysis of *A. hydrophila* and *A. caviae*)

CTAB isolation of Genomic DNA

DNA isolation from the strains of interest was performed, with minor differences, according to the cetyl-trimethylammonium-bromide (CTAB) extraction method described in Current Protocols in Molecular Biology, 1994. Strains were first grown overnight (O/N) at 37°C in 5 mL of the appropriate liquid growth media. A total of 1.5 mL liquid culture was pelleted via centrifugation before re-suspension of the pellet in 567 µL TE buffer, with the addition of 30 µL 10% SDS and 3 µL 20mg/mL proteinase K (final concentration 100 µg/mL proteinase K in 0.5% SDS) and incubated for 1.5 hr at 37°C to lyse cells. One hundred µL 5M NaCl and 80 µL CTAB/NaCl solution were subsequently added and the reaction mixture was incubated for 10 min at 65°C.

Following treatment with CTAB, 0.75 mL 24:1 chloroform/isoamyl alcohol was added and the resulting suspension was centrifuged for 10 min at max speed. 600 µL of the supernatant was pipetted into a fresh microcentrifuge tube and treated with 3 µL RNaseA for 1 h at 37^{o}C to remove any ribonucleic acid contamination. Following RNaseA treatment, an equal volume of 600 µL 25:24:1 phenol/chloroform/isoamyl alcohol was added before re-centrifuging for 10 min. The resulting supernatant was transferred to a fresh tube, where 0.6 volume isopropanol (~390 µL) was used to precipitate the DNA. DNA was re-pelleted and re-suspended in 70% ethanol to remove any residual contaminants, culminating in a final centrifugation for 5 min. The resulting DNA was dried for 1.5h in a lyophilizer before being dissolved in 100 µL TE Buffer for storage at -20^{o}C. The quality and quantity of DNA isolated was analyzed using the GeneSYS 10 Bio DNA spectrophotometer (Thermo Scientific) and 0.75% agarose gel electrophoresis. DNA stocks were made for each strain with a final DNA concentration of 50 µg/mL. Due to low DNA concentrations, strains 80, 106, 127, 133, 136, 11N, 115, 158, and SSU were re-extracted separately. Aliquots of each 50 µg/mL stock for every isolate were run on 0.75% agarose gels to confirm proper concentrations. (See appendix D for detailed procedure.)

Restriction Digest

Based on the DNA concentration established via DNA spectrophotometry, a 50 µL reaction mixture was created to digest 1 µg of DNA for each isolate. An appropriate aliquot, containing 1 µg of isolated DNA, was added to 0.5 µL 100x BSA (New England Biolabs), 5 µL 10x NEB4 (New England Biolabs), 1 µL ApaI (at 50 U/ µL, New England Biolabs), and topped off to 50 µL with Milli-Q water. Samples were incubated overnight at 37^{o}C, after which 1 µL TaqI (at 100 U/ µL, New England Biolabs) was added, and incubation was continued at 65^{o}C for 4 h. The digested fragments were heat shocked for 20 min at 80^{o}C to inactivate the restriction enzymes.

To test proper digestion conditions, strains 3, 12, and 139 were digested with ApaI and TaqI prior to examining the remaining samples. Eighteen µL of the post digestion mixture was combined with 2 µL loading buffer and run on a 0.75% agarose gel. The remaining samples were subsequently digested under the same conditions in preparation for FAFLP.

Adapter Ligation

Following the restriction digest, double strand adapters specific for each enzyme (ordered as oligonucleotides) were ligated to the restriction fragments. The ligation reaction was performed according to Janssen et al. 1996, which was a slight

modification on the original mixture described by Vos et al. 1995. A total volume of 10 µL was added to the restriction product, with the following concentrations: 0.04 µM *Apa*I adaptor, 0.4 µM *Taq*I adaptor, 1 U T4 DNA-ligase, 1mM ATP in 10 mM Tris-HAc pH 7.5, 10 mM MgAc, 50 mM KAc, 5 mM DTT, and 50 ng/µL BSA. This reaction mixture was incubated for 3 h at 37^{o}C, and diluted post-ligation to 500 µL with 10 mM Tris-HCl, 0.1 mM EDTA pH 8.0. Ligation products were stored at -20^{o}C as necessary. (See appendix D for detailed procedure)

PCR Amplification of Fragments

PCR amplification of restriction digest fragments was performed with a reaction mixture of 10 µL, comprised of 1.5 µL digested template fragments, 0.5 µL A01-6FAM labeled primer, 0.5 µL T01-6FAM labeled primer, and 7.5 µL Amplification Core Mix (included with primers).

The PCR program was initiated with a 2 min denaturing at 94^{o}C, followed by 10x cycle of: denaturation at 94^{o}C for 20 sec, annealing at [66-(n-1)]^{o}C for 30 sec (where n = the cycle number) and extension at 72^{o}C for 2 min. The reaction continued with 20x cycle of denaturing at 94^{o}C for 20 sec, annealing at 56^{o}C for 30 sec, and extension at 72^{o}C for 2 min. The program finished with the final extension at 60^{o}C for 30 min. (See appendix D for detailed procedure.)

Analysis of PCR Products

Four µL of each PCR product was combined with 6 µL of master mix in individual wells in a 96-well plate (Corning). The master mix was prepared by mixing 776µL HiDi Formamide (ABI) with 24µL each of GeneScan 500 and GeneScan 2500 TAMRA size standards (ABI). After the addition of the PCR products, a septa seal was placed on the plate and the plate was spun at low speed for 1 min. Following centrifugation, the plate was heat shocked at 95^{o}C for 5 min, placed on ice for 10 min, and spun again at low speed for 1 min before being placed in the ABI 3730xl capillary DNA sequencer.

Each plate was analyzed by using the ABI 3730xl running on the "Fragment_analysis_D_run" setting. The Fragment_analysis_D_run instrument protocol is based off of the GeneMapper36_POP7 run module, using the same Any4Dye dye set with a shorter run time of 1700 seconds per plate. (See appendix E for detailed procedure.)

Phylogenetic analysis

Output from the 3739xl sequencer was analyzed using GeneMapper 3.7 (ABI) software using a protocol from Dr. David Hawthorne (UMCP). Using GeneMapper, amplified restriction fragments (alleles) were identified for analysis and each sample was scored for the presence or absence of each allele. (See appendix G for detailed procedure.)

Statistical Analysis

The resulting tables were analyzed for similarity via the DendroUPGMA program by Garcia-Vallve et al. (http://genomes.urv.cat/UPGMA/) utilizing UPGMA and the Pearson correlation coefficient (r). Distance values were calculated using the formula: d = (1 - r) * 100. DendroUPGMA output was visualized using the program Dendroscope (Huson et al. 2007).

Table I – List of *Aeromonas* Strains for AFLP

Cluster	Species[c]	Strain	Donor Designation	Geographic Source	Source of Isolation
C[a] (10)[b]	***A. veronii***	149	ATCC 35624	ATCC/USA/MI	sputum
	bv. veronii				
H (1)	***A. hydrophila***	1	ATCC 7966	ATCC	canned milk
I (1)	***A. hydrophila***	10	AMC 6437-W	USA/MD	foot
	(arabinose -)	11	AMC 12148-E	USA/MD	fecal
		14	AMC 3276-W	USA/MD	wound
		56	AMC 3469-E	USA/MD	fecal
		64	AMC/RICE	USA/MD	knee
		66	AMC/CAMARE	USA/MD	heel
		70	AM/GALLBLADDER	USA/MD	gallbladder
		79	MOB/A12	Bangladesh	fecal
		80	MOB/A13	Bangladesh	fecal
		81	MOB/A14	Bangladesh	fecal
		82	MOB/A15	Bangladesh	fecal
		84	MOB/A17	Bangladesh	fecal
		104	L172	Sudan	fecal
		106	L206	Sudan	fecal
		120	W107	Somalia	fecal
		126	WY520	Somalia	fecal
		127	WY561	Somalia	fecal
		133	EGY 48536	Egypt/Cairo	fecal
		136	EGY 51855	Egypt/Sinai	water tank
		141	TERRY/NIH	Puerto Rico	leg
N (14)	***A. trota***	158	ATCC 49657	India	fecal
V (5a)	***A. caviae***	137	EGY AH340	Egypt/Cairo	fecal
		139	EGY W26A	Egypt/Alex	salt lake
W (5b)	***A. caviae***	140	L226	Sudan	well water
X (4)	***A. caviae***	3	ATCC 15468T	ATCC	guinea pig
		12	AMC 12338-W	USA/MD	rectal wound
		115	B101	Somalia	fecal

[a] Dr. A. Horneman's original thesis designation

[b] DNA hybridization groups (HG), or genomospecies, are indicated in parentheses under the "Cluster" column.

[c] Phenospecies designation.

Table II – List of *A. caviae* Metabolic Variants

Parent Strain	AP[a] Variant	NAP[b] Variant	WAP[c] Variant
MB3	yes	yes	yes
MB7	yes	yes	yes
MB8	yes	yes	yes
MB10	yes	yes	no
MB11	yes	no	yes
MB16 □[d]	yes	yes	no
MB16 β[e]	yes	yes	no

List of metabolic variants observed and isolated for study. Not all variants were present for every parent strain of *A. caviae*.

[a] AP = acid producing
[b] NAP = non-acid producing
[c] WAP = weak acid producing
[d] γ = γ (no) hemolysis
[e] β = β hemolysis

RESULTS

A. caviae Variant Activity on Lactose

The strains in this study included MB3 AP, MB3 NAP, MB3 WAP, MB7 AP, MB7 NAP, MB7 WAP, MB8 AP, MB8 NAP, MB8 WAP, MB10 AP, MB10 NAP, MB11 NAP, MB11 WAP, MB16 APγ, MB16 NAPγ, MB16 APβ, and MB16 NAPβ. Strains were labeled as acid producer (AP), non-acid producer (NAP), and weak acid producer (WAP) depending on their ability to produce acid from lactose. Acid producers from lactose colonies were typically small, circular, and dull pink colonies on MacConkey agar (MAC). Weak acid producing colonies on MAC tended to be circular as well, but larger in size, lighter pink, and glistening. Non-acid producing colonies were the most varied in that they were the largest in size, circular, and were glistening or dull white in appearance. In addition, NAP isolates presented with a musky odor that was not seen with AP or WAP isolates, thus suggesting metabolic differences among the three variants. β in the label represents β-hemolytic activity on blood agar and γ indicates no hemolysis.

In order to study the acid production capabilities from lactose of *A. caviae*, twenty-four isolates from various geographic sources (USA, Sudan, Somalia, Egypt, Indonesia, and Bangladesh) were characterized. Samples were previously isolated from both clinical and environmental specimens by Dr. A. Horneman in the laboratory of Dr. S. W. Joseph. Each isolate was first revived from freezer stock onto

BHI agar before it was sub-cultured onto MAC and incubated overnight at 37^{o}C. All cultures in this study were grown under ambient conditions unless otherwise stated. Plates were examined after incubation at 37^{o}C for growth, before further incubation for 4 days at room temperature (Table III). The majority of the isolates were capable of growing on MAC with various degrees of acid production from lactose, but nine isolates (6, 12, 34, 38, 54, 115, 125, 134, and 156) did not grow on the differential medium.

Strains 22, 44, 102, 132, 124, and 130 grew but did not produce acid on MAC even after 4 days. Strains 97 and 16 were negative after 18-24 h incubation but showed acid production after 4 days. Strains 8, 137 and 140 showed minimal growth after 18-24 h incubation but revealed acid production after 4 days. Strain 63 was the only acid producer after 18-24 h.

A. caviae **Tissue Cell Invasion**

Tissue cell invasion assays were performed on two sets of isolates (MB3 AP, MB3 NAP, MB10 AP, and MB10 NAP) in order to assess invasion and adhesion properties (Table IV). All isolates showed the ability to adhere, and the majority was also capable of invading T84 tissue cells. This confirms that *A. caviae* is capable of adhering to and invading tissue cell monolayers. Based on this limited sample size, it appears that there may be some differences in invasion between isolates from

different strains (MB3 appears to be more invasive than MB10) rather than between metabolic variants (AP variants exhibit similar invasion rates as NAP variants).

DNA Sample Purification

Following genomic DNA isolation from each of the various *Aeromonas* strains, 50 µg/mL genomic DNA stocks created for each sample. Aliquots from each stock were run on 0.75% agarose gels; genomic DNA bands were all approximately the same intensity (indicating similar concentrations). A sample gel is shown in Figure 1. Ladder lanes contain Bioline HyperLadder I (200 - 10,000 bp).

In order to confirm the viability of the digestion conditions, strains 3, 12, and 139 were digested with ApaI and TaqI. The gel (Figure 2) demonstrates that digestion occurs, producing a large variety of fragments. Lanes 1 and 5 contain Bioline HyperLadder I (200 - 10,000 bp).

Polyphasic Phenotyping

ONPG Utilization by *A. caviae* strains

All MB isolates were assayed for β–galactosidase activity using the ONPG disk assay (Table VII). All samples except for MB16 NAP-β, MB11 WAP, MB3 NAP, MB7 WAP, and MB8 WAP tested positive after 5 h of incubation, and all samples tested positive (yellow product) after 48 h of incubation at 35^{o}C. The positive tests indicate

that all of the MB isolates possess β-galactosidase as reflected by their ability to cleave ONPG.

Sugar Utilization by *Aeromonas* strains

The majority of strains were capable of utilizing each sugar as the sole carbon source; however, growth was very sparse with small pinhead-sized colonies (Tables VI, VII, and VIII). Isolates MB7 AP and MB8 WAP were completely unable to utilize L-arabinose, while strains 81, 106, MB8 AP, and MB10 NAP produced some growth when subcultured. Specific metabolic variants of *A. caviae* (MB3 AP, MB7 NAP, MB10 AP, MB16 APβ, and MB16 APγ) proved to be the most capable at utilizing arabinose. The majority of strains were also capable of utilizing lactose as the sole carbon source, including even all of the non-acid producing MB variants included in the study. 8WAP was the only isolate that was unable to grow even when given additional incubation time. *A. caviae* strains 63, 99, 102, 124, 129, 130, 131, and 132 (DNA HG 4, 5a, and 5b) were included in order to better characterize the ability of the species to utilize L-fucose. With the exception of strain 130, all strains were able to grow minimally on the L-fucose utilization medium. MB16 APγ and MB16 NAPγ were able to grow relatively well after subculturing at 2 days.

FAFLP

Following GeneMapper (version 3.7) analysis, a total of 137 restriction fragments (alleles) were identified for fingerprinting (Table IX). Two separate

genomic DNA samples were isolated and run for each strain, and each sample was scored independently for the presence or absence of each allele. The resulting data was analyzed by the DendroUPGMA program (Garcia-Vallve et al., 1999) and Dendroscope (Huson et al. 2007) was used to visualize the trees (Figure 3 and Figure 4).

Both sets of samples presented with three main clusters: a cluster of arabinose negative *A. hydrophila,* a cluster of MB3 and MB7 strains, and a cluster of *A. caviae strains. A. hydrophila* subsp. *dhakensis* was found to lie within the arabinose negative *A. hydrophila* cluster in both sets of data. The closest neighbors were found to be strains 70, 80, 106, 126, and 127 (arabinose negative *A. hydrophila*).

The *A. caviae* metabolic variants arising from strains MB3 and MB7 were found to cluster independently, separate of the other *A. caviae* strains included in the study. Variants derived from parent strains MB8, MB10, MB11 and MB16B and MB16Y were found to cluster indistinctly with *A. caviae* isolates identified as belonging to HG groups (genomospecies) 4, 5a, and 5b.

Table III – *A. caviae* Activity on MacConkey Agar

Strain #	Location isolated	Source	MacConkey	
(n = 24)			O/N	>4 days
22	USA/MD	fecal	NAP[a]	NAP
44	USA/MD	blood	NAP	NAP
63	USA/DC	fecal	AP[b]	AP
97	Sudan	fecal	NAP	WAP[c]
99	Sudan	fecal	WAP	WAP
102	Sudan	well water	NAP	NAP
132	Sudan	fecal	NAP	NAP
116	Somalia	fecal	NAP	WAP
124	Somalia	rectal	NAP	NAP
129	Somalia	fecal	WAP	WAP
130	Somalia	fecal	NAP	NAP
131	Somalia	fecal	WAP	WAP
8	Jakarta	fecal	low growth	WAP
137	Egypt/Cairo	fecal	low growth	WAP
140	Sudan	well water	low growth	WAP
12	Somalia	fecal	No growth	No growth
125	Somalia	fecal	No growth	No growth
6	USA/NY	blood	No growth	No growth
34	USA/MD	fecal	No growth	No growth
38	USA/MD	fecal	No growth	No growth
54	USA/MD	horse/foot	No growth	No growth
115	Somalia	fecal	No growth	No growth
134	Egypt/Cairo	water tank	No growth	No growth
156	Bangladesh	fecal	No growth	No growth

Strains are organized by their origin of isolation.

[a] NAP = non-acid production

[b] AP = acid production

[c] WAP = weak acid production

Table IV – *A. caviae* Tissue Cell Invasion and Cell Association[a]

Strain		AP[b] Invasion	NAP[c] Invasion	AP Ca[d]	NAP CA
MB3	Trial 1	0	0	1.99E+07	4.80E+06
	Trial 2	1.85E+06	N/A[e]	1.67E+07	N/A
	Trial 3	1.30E+06	N/A	1.39E+07	N/A
MB10	Trial 1	2.00E+05	1.70E+05	1.32E+07	3.97E+07
	Trial 2	6.70E+05	1.20E+05	1.37E+07	1.87E+07
	Trial 3	5.50E+05	0	1.71E+07	1.13E+07

[a] Initial inoculum for each well was 1 x 10^8 for a MOI of 100:1
[b] AP = acid producing
[c] NAP = non-acid producing
[d] CA = cell association
[e] N/A = experiments were not conducted

Table V – ONPG Hydrolysis

Strain	Isolate		5 hr	48 hr
MB3	AP[a]		+	+
	WAP[b]		+	+
	NAP[c]		N/A (clear)	+
MB7	AP		+	+
	WAP		N/A (clear)	+
	NAP		+	+
MB8	AP		+	+
	WAP		N/A (clear)	+
	NAP		+	+
MB10	AP		+	+
	NAP		+	+
MB11	WAP		N/A (clear)	+
	NAP		+	+
MB16	AP-γ[d]		+	+
	NAP-γ		+	+
	AP-β[e]		+	+
	NAP-β		N/A (clear)	+

+ = visible yellow product

[a] AP = acid producing

[b] WAP = weak acid producing

[c] NAP = non-acid producing

[d] Y = γ (no) hemolysis

[e] B = β hemolysis

Table VI – L-arabinose Utilization

Strain	O/N	2 day restreak		Strain	O/N	2 day restreak
1	+/-	+/-		MB3 AP[a]	+	+
10	+/-	+/-		MB3 NAP[b]	+/-	+/-
11	+/-	+/-		MB3 WAP[c]	+/-	+/-
14	+/-	+/-		MB 7AP	-	-
56	+/-	+/-		MB7 NAP	+	+/-
64	+/-	+/-		MB7 WAP	+/-	+/-
66	+/-	+/-		MB8 AP	-	+/-
70	+/-	+/-		MB8 NAP	+/-	+/-
79	+/-	+/-		MB8 WAP	-	-
80	+/-	+/-		MB10 AP	+	+
81	-	+/-		MB10 NAP	-	+/-
82	+/-	+/-		MB11 NAP	+/-	+/-
84	+/-	+/-		MB11 WAP	+/-	+/-
104	+/-	+/-		MB16 APβ[d]	+	+
106	-	+/-		MB16 APγ[e]	+	+
120	+/-	+/-		MB16 NAPβ	+/-	+/-
126	+/-	+/-		MB16 NAPγ	+/-	+/-
127	+/-	+/-				
133	+/-	+/-				
136	+/-	+/-				
141	+/-	+/-				
DHA	+/-	+/-				
SSU	+/-	+/-				

+ = full growth; +/- = low growth; - = no growth

[a] AP = acid producing

[b] NAP = non-acid producing

[c] WAP = weak acid producing

[d] B = β hemolysis

[e] Y = γ (no) hemolysis

Table VII –Lactose Utilization

Strain	O/N	2 day restreak		Strain	O/N	2 day restreak
1	+	+		MB3 AP[a]	+	+/-
10	+/-	+/-		MB3 NAP[b]	+	+/-
11	+/-	+/-		MB3 WAP[c]	+	+/-
14	+/-	+/-		MB 7AP	-	+
56	+/-	+/-		MB7 NAP	+	+
64	+/-	+/-		MB7 WAP	+	+
66	+/-	+/-		MB8 AP	-	+
70	+/-	+/-		MB8 NAP	+	+
79	+/-	+/-		MB8 WAP	-	-
80	+/-	+/-		MB10 AP	+	+
81	+/-	+/-		MB10 NAP	+/-	+
82	+/-	+/-		MB11 NAP	+	+
84	+/-	+/-		MB11 WAP	+	+
104	+/-	+/-		MB16 APβ[d]	+	+
106	-	+/-		MB16 APγ[e]	+	+
120	+/-	+/-		MB16 NAPβ	+	+
126	+/-	+/-		MB16 NAPγ	+	+
127	+/-	+/-				
133	+/-	+/-				
136	+/-	+/-				
144	+/-	+/-				
DHA	+/-	+/-				
SSU	+/-	+/-				

+ = full growth; +/- = low growth; - = no growth

[a] AP = acid producing

[b] NAP = non-acid producing

[c] WAP = weak acid producing

[d] B = β hemolysis

[e] Y = γ (no) hemolysis

Table VIII – L-fucose Utilization

Strain	O/N	2 day restreak		Strain	O/N	2 day restreak
10	+/-	+/-		MB3 AP[a]	+/-	+/-
11	+/-	+/-		MB3 NAP[b]	+/-	+/-
14	+/-	+/-		MB3 WAP[c]	+/-	+/-
56	+/-	+/-		MB 7AP	+/-	+/-
64	+/-	+/-		MB7 NAP	+/-	+/-
66	+/-	+/-		MB7 WAP	+/-	+/-
70	+/-	+/-		MB8 AP	+/-	+/-
79	+/-	+/-		MB8 NAP	+/-	+/-
80	+/-	+/-		MB10 AP	+/-	+/-
81	+/-	+/-		MB10 NAP	+/-	+/-
82	+/-	+/-		MB11 NAP	+/-	+/-
84	+/-	+/-		MB11 WAP	+/-	+/-
104	+/-	+/-		MB16 APβ[d]	+/-	+/-
106	+/-	+/-		MB16 APγ[e]	+/-	+
120	+/-	+/-		MB16 NAPβ	+/-	+/-
126	+/-	+/-		MB16 NAPγ	+/-	+
127	+/-	+/-				
133	+/-	+/-		63	+/-	+/-
136	+/-	+/-		99	+/-	+/-
141	+/-	+/-		102	+/-	+/-
DHA	+/-	+/-		124	+/-	+/-
SSU	+/-	+/-		129	+/-	+/-
				130	-	-
				131	+/-	+/-
				132	+/-	+/-

+ = full growth; +/- = low growth; - = no growth

[a] AP = acid producing

[b] NAP = non-acid producing

[c] WAP = weak acid producing

[d] B = β hemolysis

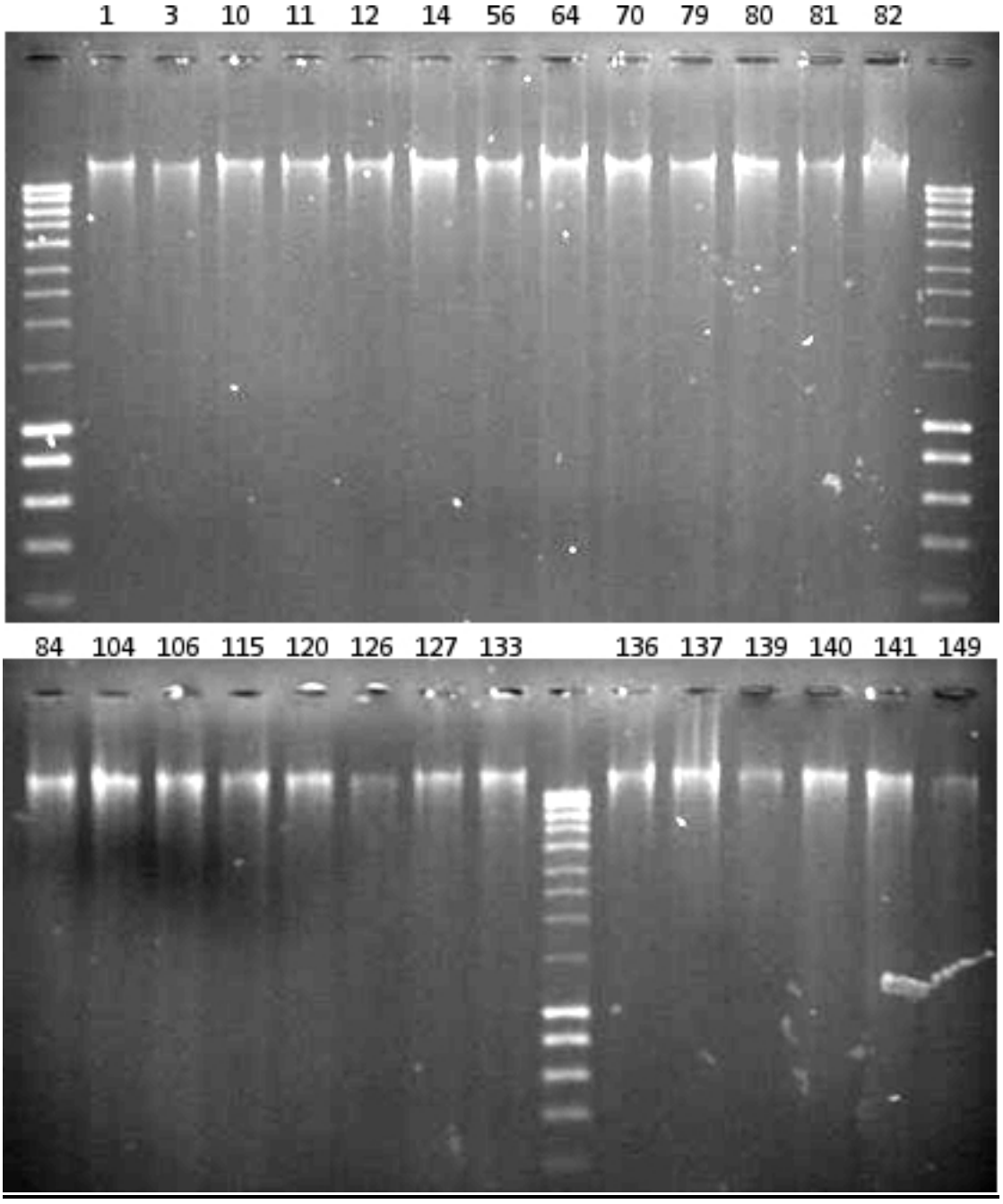
[e] Y = γ (no) hemolysis

Table IX - GeneMapper Identified Alleles

Allele	Size (bp)		Allele	Size (bp)		Allele	Size (bp)
1	56.82		47	103.45		93	153.46
2	57.54		48	104.73		94	156.86
3	58.54		49	105.9		95	160.12
4	60.11		50	106.78		96	160.97
5	62.25		51	108.66		97	162.34
6	63.19		52	109.3		98	164.32
7	63.87		53	109.83		99	165.76
8	64.84		54	110.26		100	166.52
9	65.86		55	111.07		101	167.32
10	66.49		56	112.83		102	168.22
11	67.3		57	113.65		103	168.93
12	68.81		58	114.54		104	171.23
13	71.54		59	115.45		105	172.6
14	72.23		60	116.21		106	174.15
15	73.58		61	117.26		107	175.22
16	74.41		62	117.87		108	176.17
17	75.67		63	118.58		109	177.77
18	77.32		64	120.05		110	178.7
19	78.57		65	121.39		111	179.74
20	78.98		66	122.29		112	182.29
21	80.19		67	123.24		113	187.64
22	80.96		68	124.19		114	190.42
23	81.96		69	126.32		115	191.58
24	83.4		70	127.08		116	192.99
25	83.89		71	127.58		117	197.98
26	84.32		72	128.22		118	198.93
27	85.08		73	128.82		119	200.38
28	86.09		74	129.92		120	200.92
29	86.57		75	130.77		121	201.55
30	87.8		76	132.02		122	202.79
31	88.46		77	133.49		123	208.68
32	89.14		78	134.75		124	210.53
33	90.54		79	135.27		125	211.96
34	90.92		80	136.29		126	213.78
35	91.8		81	137.17		127	214.51
36	93.13		82	138.83		128	215.19
37	93.71		83	139.61		129	216.67
38	94.39		84	140.67		130	225.25
39	95.03		85	141.69		131	225.78
40	95.77		86	143.15		132	226.53
41	96.99		87	145.13		133	227.82
42	97.7		88	145.92		134	234.22
43	98.4		89	146.67		135	236.17
44	99.56		90	147.9		136	248.47
45	100.13		91	149.88		137	259.68
46	102.46		92	151.11			

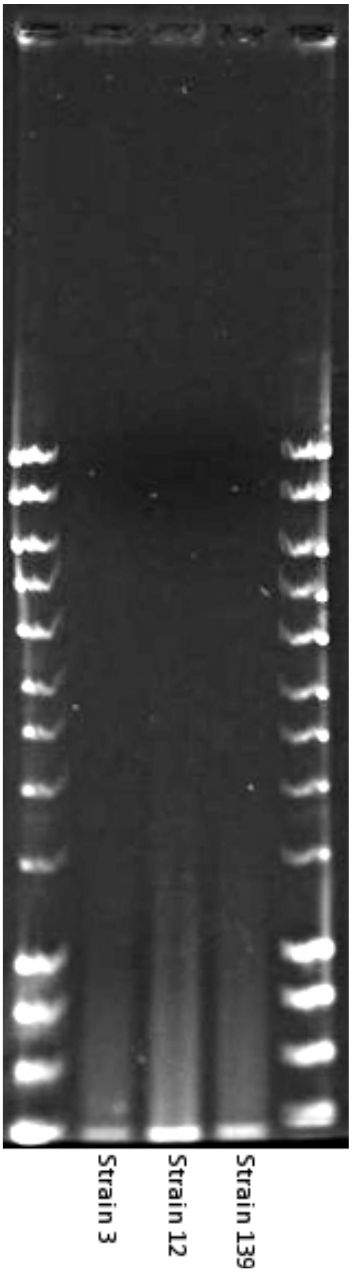
Amplified restriction fragments (alleles) identified using GeneMapper.

Figure I – Confirmation of DNA Stock Concentrations



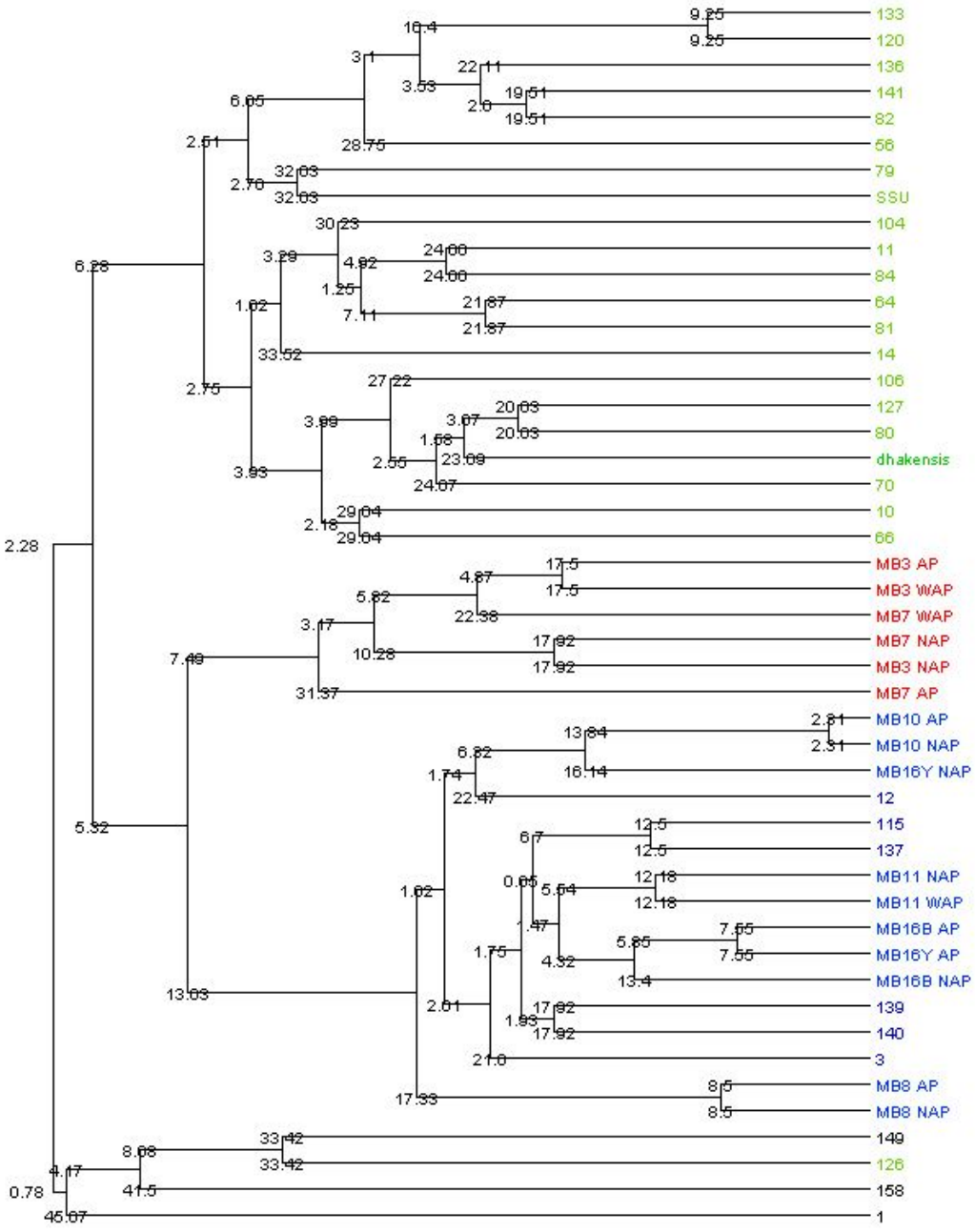
Determining the concentrations of genomic DNA stocks. Each lane is labeled with the corresponding strain's genomic DNA stock. The three ladder lanes contain Bioline HyperLadder I (200 - 10,000 bp).

Figure II – Confirmation of Digestion Procedure



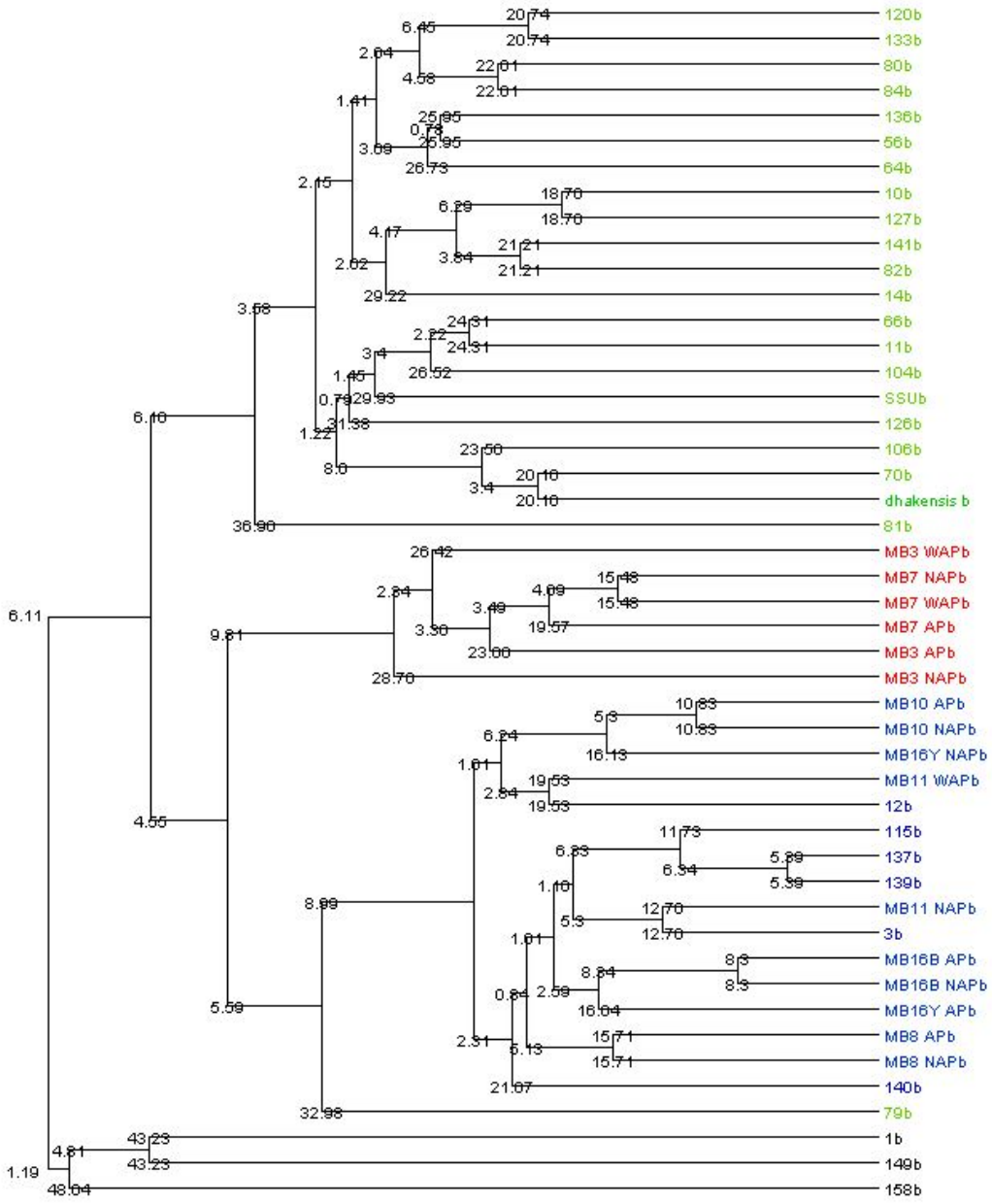
Confirmation of the viability of the digestion conditions. Strains 3, 12, and 139 were run in lanes 2, 3, and 4 respectively. Lanes 1 and 5 contain Bioline HyperLadder I (200 - 10,000 bp).

Figure III – Sample A Dendrogram



Dendrogram constructed using UPGMA with Pearson's Correlation Coefficient (r). Distance values were calculated using the formula: d = (1 - r) * 100. Samples in green are arabinose negative isolates of *A. hdyrophila*; independently clustering variants of *A. caviae* are in red; strains in blue are various *A. caviae*; strains in black are various nonrelated reference strains of *Aeromonas*.

Figure IV – Sample B Dendrogram



Dendrogram constructed using UPGMA with Pearson's Correlation Coefficient (r). Distance values were calculated using the formula: d = (1 - r) * 100. Samples in green are arabinose negative isolates of *A. hdyrophila*; independently clustering variants of *A. caviae* are in red; strains in blue are various *A. caviae*; strains in black are various nonrelated reference strains of *Aeromonas*.

DISCUSSION

A. caviae Variant Identification

The phenospecies *A. caviae* includes organisms belonging to hybridization groups (genomospecies) 4, 5A, and 5B. While some environmental isolates are capable of producing acid from lactose utilization, others are not. The metabolic variants in this study were originally isolated from pure non-lactose fermenting cultures that were streaked on MacConkey agar (MAC), incubated at 37^{o}C overnight, and were left at room temperature for 4 days. After 4 days, it was discovered that each plate contained discrete acid producing (AP) colonies as well as the expected non-acid producing (NAP) colonies.

It is suggested that the production and accumulation of acidic products by AP colonies may reduce the rate of growth, resulting in smaller colonies when compared to NAP. The distinction between acid production from lactose and lactose fermentation should also be noted. It is possible to produce acidic metabolites from oxidative methods of metabolism rather than by fermentation (ex. *Neisseria* spp.; Knapp and Rice, 1995). In this study, the exact metabolic differences between AP and NAP isolates are yet unknown; therefore, the assumption that AP colonies are capable of lactose fermentation cannot be made.

When AP colonies were subcultured, the phenotype remained AP with no reversions observed. Additionally, colony morphology was found to vary significantly when comparing AP with NAP on MacConkey agar. AP colonies were circular, smaller, and dull in appearance when compared to the larger NAP colonies. The differences in colony morphology, in addition to the persistence of the phenotype, suggest that the variance in sugar fermentation is not due just to spontaneous changes in gene expression. In order to further investigate this phenomenon, twenty-four additional A. caviae isolates were selected as reference strains, incubated under identical conditions, and examined at 18 – 24 h and after 4 days of incubation at room temperature. The reference isolates studied were previously obtained as both clinical and environmental specimens from varied geographical locations.

While the majority of the tested A. caviae isolates were capable of growing on MacConkey agar with various degrees of acid production from lactose, none of the plates contained colonies with more than one phenotype or type of colony morphology. This finding suggested that metabolic variation is not normally seen within the A. caviae population on a single plate and also that the initial MB cultures, while pure, contained variants at the subspecies level.

It was surprising that nine isolates (6, 12, 34, 38, 54, 115, 125, 134, and 156) produced no growth on MacConkey agar; these nine strains also grew poorly on BHI. The isolates belonged to a variety of DNA hybridization groups (4, 5a, and 5b), suggesting that the issue was not restricted to a group of closely related strains. Freezer stocks for these isolates were made approximately twenty years ago, so it is possible that the stocks were weakened following long term storage at -80oC. Additionally, depending on the strain of *A. caviae*, growth on differential medium can be significantly hindered when compared to growth on a nonselective medium such as blood agar or BHI (Desmond and Janda, 1986). It is plausible that the combination of poor growth on BHI and the subsequent subculture onto a selective medium resulted in no noticeable growth.

Tissue Cell Invasion

It has been shown in *Salmonella spp*. (Bacci et al. 2006) that differences in metabolic capability between species can reflect differences in their virulence capabilities. Therefore, it was of interest whether differences in acid production from lactose would similarly reflect a difference in virulence between AP and NAP *A. caviae* strains. A quantifiable measure of the ability of *A. caviae* to invade tissue cells could reflect the overall virulence of the organism.

Aeromonas is known to possess a variety of virulence factors that could contribute to causing disease. However, the exact mechanism used by *Aeromonas* to cause intestinal tissue damage is still unknown, mainly due to lack of an animal model that could reproduce the gastroenteric disease seen in humans (Thorley et al. 1996; Joseph and Carnahan. 2000). Other researchers have demonstrated that different types of *Aeromonas* infection involve complex pathogenic mechanisms, including invading and penetrating the intestinal epithelial monolayer (Thorley et al. 1996; Kirov 1997; Rocha-de-Souza et al. 2003)

A method for studying tissue cell invasion was first described by Mehlman et al. in 1982 for use with *E. coli*; the environment of the intestinal lining was simulated by a monolayer of HeLa cells on the bottom of a six-well plate. In the following years, subsequent trials have shown that the method is reliable and valid as a virulence assay for a variety of pathogenic enteric organisms, including *Aeromonas* (Couto et al. 2007).

During an invasion assay, a monolayer of tissue cells (a layer of T84 cells were used in this experiment) is exposed to a known number of pathogens and the system is incubated for three hours to allow for invasion. After a suitable length of time, the tissue cells are washed and treated with the antibiotic gentamicin, chosen specifically because it is not taken up by eukaryotic cells. Any bacteria that were

able to invade and penetrate the cells survive the antibiotic treatment, while those that are still free floating or adhering to the exterior of the host cells will not. Following gentamicin treatment, the tissue cells are then lysed and the resulting cell lysate is serially diluted and plated in order to quantify the number of bacteria that were able to invade. This assay, however, hinges on the ability of the pathogen to invade the host cells; the assay will not yield any useful results If the pathogen produces cytotoxic (causing extensive damage to the intestinal epithelium) or cytopathic (does not damage intestinal crypts or microvilli but stimulates secretion of water and salt) effects as its primary method of pathogenesis.

Previously, Rocha-de-Souza et al. demonstrated that *Aeromonas* strains are capable of adhering to both polarized and nonpolarized Caco-2 cells. More recently, Couto et al. described an environmental isolate of *A. caviae* that was able to invade Caco-2 cells within 90 minutes (it was not reported whether the Caco-2 monolayer was polarized or nonpolarized). Couto et al. also showed that certain strains of *A. caviae* are capable of producing cytotoxic and cytopathic effects; however, they required a minimum of 2 hours and 24 hours, respectively (Couto et al. 2007).

The limited results obtained thus far (Table IV) suggest that at 3 hours, invasion does still occur despite the potential production of cytotoxin. It is interesting to note that with isolate MB10, there does not seem to be a large

difference in invasive ability between AP and NAP isolates, while there appear to be more significant differences in adhesion between the two metabolic variants of MB3.

For further investigation, more strains must be studied. The current data confirms that *A. caviae* is capable of invading T84 tissue cells; however, it is still unclear whether or not there is a significant difference in invasion and adhesion ability between acid producing and non-acid producing variants. The experiments in this study were performed at an MOI of 100:1 based on suggested numbers of aeromonads by Couto et al.; however, additional trials should be run to confirm that it is appropriate (countable number of colonies on dilution plates) for *A. caviae.* It is also important to conduct invasion assays with varied incubation times between 90 minutes and 3 hours to confirm that the invasion results are occurring independently of any cytotoxic or cytopathic effects. Finally, it would also be interesting to study another potential aspect of virulence by conducting cytotoxic assays to assess cytotoxin production.

Polyphasic Phenotyping

ONPG and Lactose Utilization by *A. caviae* Strains

Ortho-Nitrophenyl-β-galactoside (ONPG) is a colorimetric substrate used to detect β-galactosidase activity. β-galactosidase is required for lactose utilization,

and if the enzyme is present, it hydrolyzes the normally colorless ONPG molecule into galactose and ortho-nitrophenol. Ortho-nitrophenol has a yellow color that is visible to the naked eye, and the intensity of the color produced can be used as a measure of the enzymatic rate. ONPG mimics lactose and is hydrolyzed by β-galactosidase; however, it does not induce expression of the lac operon. Therefore, organisms must be cultured on lactose rich media prior to ONPG testing in order for β-galactosidase to be transcribed. When the MB isolates were assayed for β–galactosidase activity using the ONPG disk assay (Table VII), all samples tested positive, indicating that the isolates possessed β-galactosidase as reflected by their ability to cleave ONPG.

In order for *Aeromonas* to utilize lactose, it must be transported across the cell membrane by lactose permease before β-galactosidase hydrolyzes the lactose molecule to glucose and galactose. By contrast, the ONPG reagent is able to diffuse through the membrane without the aid of a transporter. It was very surprising to note that, while growth was minimal, all of the non-acid producing MB variants in the study were capable of utilizing lactose as the sole carbon source for growth. Therefore, the ONPG results (Table VII) suggest that the NAP isolates are deficient in a step in lactose metabolism prior to β-galactosidase, namely lactose permease. It was interesting to note that the MB variants also appeared to be capable of utilizing

L-arabinose and L-fucose; however, there did not appear to be significant differences in the ability to utilize the two sugars between AP and NAP variants.

On MAC, organisms that are incapable of utilizing lactose are able to break down peptone instead; however, the lactose utilization minimal media contains no other carbon source except lactose. In order to discount the possibility that growth was occurring using endogenous metabolites remaining from the rich media (BHI) used to revive the strains from freezer stock, samples were subcultured following 2 day incubation on the sugar utilization medium. Growth was observed after subculturing; however, there appeared to be variations in colony size between AP and NAP cultures. At this point, while there are clear metabolic differences between AP and NAP isolates, the exact mechanism causing these differences is still unknown.

Future work should examine the possibility that lactose permease might be deficient but still functional in NAP isolates. In the current study, lactose utilization plates were examined at 24 h intervals and scored for growth. However, the exact amount of growth was only generally classified: growth (+), low growth (+/-), and no growth (-). Additional attention must be given to the colony size, morphology, and speed of growth; if NAP presents with smaller or slower growing colonies when

compared to AP isolates, this may suggest a deficient rather than inactivated lactose permease.

Sugar Utilization by *A. hydrophila* strains

In order to characterize the new subspecies *A. hydrophila* subsp. *dhakensis*, Huys et al. analyzed 152 phenotypic characteristics; it was found that eight specific tests: utilization of L-arabinose, L-fucose, methyl a-d-mannoside, urocanic acid, *cis*-aconitate, DL-aspartate, caprate, and acid production from L-arabinose, could be used to distinguish *A. hydrophila* subsp. *dhakensis* from DNA HG 1 *A. hydrophila* (Huys et al. 2002). Previous work with a large group of HG 1 *A. hydrophila* corroborated the results obtained by Huys et al. from the utilization of L-arabinose, utilization of urocanic acid, and production of acid from L-arabinose (Abbott et al. 1992; Hänninen 1994; Kämpfer and Altwegg 1992), therefore, it was suggested that these three tests be considered the major differential tests used for the identification of the subspecies *dhakensis*. *A. hydrophila* subsp. *dhakensis* was defined as unable to utilize L-arabinose, non-acid producing on L-arabinose, and able to utilize urocanic acid as its sole carbon source.

Previous work by Carnahan and Joseph had described a genotypically distinct group of arabinose negative *A. hydrophila*, which like *A. hydrophila* subsp. *dhakensis*, did not produce acid from L-arabinose (Carnahan and Joseph 1993). It was suspected that these strains potentially belonged to the subspecies *dhakensis*;

therefore, a panel of arabinose negative *A. hydrophila* isolates was cultured on the L-arabinose and also an L-fucose utilization medium. Like with the lactose utilization assay described above, samples were subcultured following 2 days of incubation on the sugar utilization medium to discount the possibility that growth was occurring using endogenous metabolites remaining from BHI used to revive the samples from freezer stock.

Huys et al. and Martinez-Murcia et al. had found that *A. hydrophila* subsp. *dhakensis* was incapable of utilizing L-arabinose. Thus, it was contrary to expectations that all arabinose negative (non-acid producing) isolates (strains 10, 11, 14, 56, 64, 66, 70, 79, 80, 81, 82, 84, 104, 106, 120, 126, 127, 133, 136, 141, and *dhakensis*) were able to grow on the minimal media and grew at approximately the same rate as the arabinose positive (acid producing) type strain of *A. hydrophila* (strain 1). Urocanic acid was unavailable for this study; therefore, utilization of L-fucose was included instead. Huys et al. found *A. hydrophila* subsp. *dhakensis* to be incapable of L-fucose utilization, but again, it was surprising to note that all arabinose negative strains in this experiment (including subsp. *dhakensis*) were able to grow on minimal L-fucose media. Finally, the arabinose negative strains were also streaked onto excess lactose utilization media from earlier experiments. It was interesting to observe that HG 1 *A. hydrophila* grew quite well on this medium, while arabinose negative isolates were only capable of minimal growth.

It is difficult to explain how results from this study seemingly contradict published results. A possible explanation may lie in a difference of methodology; the sugar utilization protocol used in this study was obtained from a collaborator with Dr. Martinez-Murcia rather than from the laboratory of Dr. Huys. Additionally, growth was generally minimal and so plates were generally incubated for 48 hours before observation. The presence of small pinhead colonies by 48 hours, while minimal, was still scored as a positive test and the isolate was considered able to utilize the sugar. Huys et al. also did not specify when (following how long of an incubation) and how they scored growth (presence/absence or if amount of growth was taken into account). At the present, it is unknown if the two sets of data are comparable and additional trials must be completed with these strains under more standardized conditions for concrete conclusions to be drawn.

If additional L-fucose and L-arabinose utilization data continues to prove inconclusive, previous work by Joseph and Carnahan suggest additional phenotypic tests that may aid in the identification of additional "*dhakensis*" strains. Arabinose negative strains, like subsp. *dhakensis,* were found to have a greater frequency of acid produced from salicin, greater resistance to ceftazidime, imipenem, and cefoxitin when compared to arabininose positive strains. These assays have yet to be used to compare "*dhakensis*" with arabinose positive *A. hydrophila* and may add

to the panel of tests first described by Huys et al. for the identification of subsp. *dhakensis.*

FAFLP

In the past, DNA/DNA hybridization was considered the gold standard for species classification in Aeromonas taxonomy. Due to repeatability issues (high variability with results) and the complexity of the protocol, replacement techniques and approaches have been sought. FAFLP was chosen for this study over other molecular techniques for its appropriate resolution and reproducibility: random amplification of polymorphic DNA (RAPD) analysis has a high amount of variability even within a subspecies, and pulsed-field gel electrophoresis was found to lack resolution past the species level (Huys et al. 1996). Ribosomal DNA sequence polymorphisms (ribotyping) or 16s rRNA restriction fragment analysis are also limited in their sensitivity due to lower number of bands available for analysis when compared to AFLP.

The laboratory of Dr. David Hawthorne (UMCP) regularly performs FAFLP experiments and graciously shared their FAFLP protocol; however, samples were analyzed on the ABI 3730xl DNA sequencer using the Fragment_analysis_D module instead of the AFLP_Long module suggested by the Hawthorne protocol. When Fragment_analysis_D was compared to the AFLP_Long module, there were only a few minor changes: 1.5 injection voltage instead of the AFLP_Long 1.6, 180 for 1st

and 2nd readout time instead of 200 (described in the manual as the interval of time for data points to be produced), 5 for current stability instead of 3 (ABI default setting was 5 ; current stability is the maximum allowed current variation, anything greater is attributed to bubbles in the sample and causes an abort), and most importantly, 1700 instead of 3000 seconds for run length.

Based on the 3730xl instruction manual, the differences in the run modules are negligible except for the adjustment to the run length. The Hawthorne lab suggested the longer 3000 second run time to allow for better separation of larger pieces (up to 1 kb in length). By comparison, this study utilizes AFLP to look at *Aeromonas* isolates. Due to the genome size and restriction enzymes used, shorter fragments approximately 35-500 bp in length are produced. While the AFLP_Long module could theoretically be used, running things for a longer period results in distortion of the spacing between smaller fragments (< 100 bp in size) and as a result, the long run length was not ideal for this study.

In order to create dendrograms for genotypic analysis, the un-weighted pair group method with arithmetic mean (UPGMA) was used with 100 bootstrap replicates. The tree was created with the reference strains from Table I in order to help visualize the relationship among the metabolic variants; the bootstrap replicates were conducted as part of the analysis to test the tree's reliability. The

algorithm analyzes the amplified fragment matrix when constructing a rooted tree; at each step, the nearest two clusters are combined into a higher-level cluster. Pearson's correlation coefficient is used as part of the algorithm to measure the degree of association (positive or negative) between each strain.

UPGMA was chosen for this study due to its relative ease of use, and in order to confirm the results of Huys et al. 2002, who also used this method to propose *A. hydrophila* subsp. *dhakensis* as a new subspecies. It should be recognized that this method of analysis assumes a constant rate of nucleotide substitution throughout the evolutionary history of the taxa, which may not be applicable for all cases. As a result, for more detailed phylogenetic work, UPGMA is used to produce guide trees prior to more sophisticated phylogenetic algorithms; in this case, UPGMA provides enough resolution to draw conclusions at the subspecies level.

While FAFLP results have been shown to be highly reproducible (Janssen et al. 1996; Huys et al. 1996), there were differences in the dendrograms produced by the two trials. Despite the differences in the exact order, all of the MB3 and MB7 isolates clustered together, with no close neighbors. The remaining MB8, MB10, MB11 and MB16 isolates clustered together with 3, 12, 115, 137, 139, 140 (*A. caviae* DNA HG 4, 5a, 5b) and *A. hydrophila* subsp. *dhakensis* clustered closely with 70, 80, 106, 126, and 127 (arabinose$^-$ *A. hydrophila*). When comparing MB3/MB7 cluster,

the evolutionary distances (31.37 for sample A, 28.76 for sample B) were significantly less than the distances between species *A. hydrophila*, *A. veronii*, and *A. trota* (each of which was equal or greater to 41.5 for both trials). While there is not enough evolutionary distance to be considered a new species, the separate clustering suggests that MB3 and MB7 may belong to their own subgroup of *A. caviae*. Additional differential metabolic testing to identify a unique metabolic profile must be performed before this subgroup can be defined as a new subspecies.

With regards to *A. hydrophila* subsp. *dhakensis*, the results seem to validate the work by Huys et al.; the arabinose negative strains of *A. hydrophila* are relatively distinct from the arabinose positive strains of *A. hydrophila* HG 1. Additionally, the arabinose negative strains tended to be relatively equidistant from each other with no tight clustering within the group. However, these strains, while evolutionarily distinct from HG 1, are more related with slightly smaller distance values (37.9 for A, 37.0 for B) than the values generated for distinct species of *Aeromonas* (>= 41.5). This suggests that the original proposal to classify these strains as a subspecies rather than species based on FAFLP results was conservative, but if additional genetic or phenotypic differences were reported, it could lead to a bolder classification as the novel species *A. dhakensis.*

Conclusions

While the metabolic data remains inconclusive, the FAFLP results do suggest that MB3 and MB7 may belong to a new subspecies of *A. caviae*; MB 8, MB10, MB11, MB16, and MB16 appear to belong to groups previously established through DNA-DNA hybridization. It is interesting to note that it was hypothesized that there would be differences between *A. caviae* metabolic variants; however, it appears that two out of the seven original strains potentially belong to their own subspecies instead. The exact mechanism for the differences in acid production from lactose remains unclear, as the phenomenon cannot be explained solely through variability in gene expression. Additional studies to further characterize the MB3 and MB7 variants, including metabolic and virulence assays, must be performed prior to defining the cluster as a new subspecies of *A. caviae*.

Similarly, *A. hydrophila* subsp. *dhakensis* does appear to be properly classified, at least via FAFLP, as a subspecies of *A. hydrophila* rather than a new species of *Aeromonas*. Future studies may suggest additional differences between subsp. *dhakensis* and HG 1 *Aeromonas* that may lead to a reclassification; however, the precedent in that case would be to elevate subsp. *dhakensis* to a novel species rather than the use of *A. aquariorum*, as has been championed by Martinez-Murcia et al. (Martinez-Murcia et al. 2008a)

APPENDIX A

Invasion Assay

~1 week in advance

- Seed ~2.0 x 10^5 T84 cells (using Stein lab cell line) per well on a 6 well tissue culture plate.
- Grow @ 37°C O/N with 5% CO_2 and humidity

24 hours in advance

- Streak invasion bacteria onto appropriate media and incubate appropriately O/N
- Pour at least nine 9-cm plates (with LB or TSA) and prepare about nine 900 uL (of BHI broth) dilution tubes for each well of T84 cells. Place in incubator O/N to check for contamination.

Next day (work inside hood)

Before starting:

1. Turn on blower; leave running for 15 minutes before use.
2. Turn on Klett machine, needs ~15 minutes to warm up.
3. Place invasion media bottles in 37°C water bath to warm up before use.
4. After 15 minutes, spray interior surfaces of hood with 70% alcohol and wipe clean.
5. Wipe invasion media bottles clean with the alcohol mixture, place in hood.

- Aspirate the media (using a yellow tip inside a reversed blue tip for gentle suction) from the tissue cells.
- Add 2 mL of Invasion Media (DMEM with FBS, but no antibiotics) to each well and rock gently. Aspirate the media; this is the first wash. Repeat 4 more times. Leave the 2 mL of media in each well after the final wash.

- Aliquot 4 mL of 0.85% saline to each Klett tube (one Klett tube per strain).
- Using a sterile swab, suspend bacteria in the saline and adjust concentration until a Klett reading of 100 is obtained (resulting in 2.0E8 CFU/mL concentration for *A. caviae*)
- Serially dilute cells with Invasion Media to appropriate MOI (multiplicity of infection, 100 bacteria : 1 tissue cell for *A. caviae*).
- Add 1 mL of the appropriate diluted stock to the 2 mL of Invasion Media already in each well. The invasion starts at this point.
- Incubate at 37°C with 5% CO_2 for 3 hours.

Following invasion (work outside of hood)

- After 3 hours, aspirate media (as described above) from the invasion wells only.
- Add 1 mL 200 ug/mL gentamicin in DMEM culture media and 2 mL of Invasion Media to each invasion well. This step does not apply to the cell-associated wells.
- For cell-associated wells, add 3 mL of invasion media.
- Incubate for 2 h at 37^{o}C with 5% CO_2 to allow the gentamicin to kill all cell surface attached bacteria.

- Take 1 mL of the media from each invasion well and plate 100 uL to ensure that there are no surviving free floating bacteria in the "supernatant".
- Wash each well 5x with 1x PBS (as described above with Invasion Media); aspirate the remaining wash from each well.
- Add 1 ml of 0.1% Triton X to each well to lyse the tissue cells.
- Incubate at 37^{o}C with 5% CO_2 for 15 minutes to allow the Triton X to work.

- Use a cell scraper to loosen and concentrate the lysate as much as possible. Using a micropipet, transfer the cell lysate from each well into a separate microcentrifuge tube. Pipet the solution in the tube up and down 20x to break up any cell clumps.
- Make 10^{-3} through 10^{-5} dilutions of the cell lysates, and pipet 100 uL of each dilution onto plates and spread.
- Incubate at 37^{o}C with 5% CO2 O/N. Count bacterial colonies the next day.

APPENDIX B

ONPG Disk Detection for Presence of β-Galactosidase

BD BBL Taxo ONPG disks – VWR catalog #90002-222

Objective:

This test is used to demonstrate the presence or absence of the enzymes β-galactosidase and lactose permease using the substrate ortho-nitrophenyl-β-D-galactopyranoside (ONPG). Since the enzymes are induced in the presence of lactose, the organism to be tested should be grown on media high in lactose (lactose broth) for best results (according to manufacturer's instructions).

Reagents:

ONPG disks (store refrigerated between -20 and 8^{o}C)
Sterile saline (or TSB, 0.5 mL for each sample to be tested)
Sterile small test tubes
10 µL bacteriology loop
Sterile graduated Pasteur pipette

Procedure:

24 hours prior

1. Streak the organisms to be tested on TSI agar. Incubate overnight at appropriate growing conditions

For each sample to be tested

1. Place an ONPG disk into a sterile tube and add 0.5 mL sterile saline or TSB.
2. Heavily inoculate the tube with a loopful of the test isolate. A heavy inoculum is necessary to obtain a high concentration of enzyme.
3. Incubate at 35^{o}C for 4 to 6 hours.

Controls

Positive: *E. coli* (ATCC 25922)
Negative: *M. morganii* (ATCC 8019)

Interpretation

- Positive for β-galactosidase: yellow color within 4-6 hours or following O/N incubation.
- If colorless at 4 hours, incubate O/N before discarding as negative for β-galactosidase

APPENDIX C

Carbohydrate Utilization Media

Objective:
This differential minimal media is used to test for carbohydrate utilization.

Reagents (1L):
15 g agar (Difco)

2 g of the appropriate carbohydrate carbon source (ex: L-arabinose, L-fucose, or lactose)

6.1 g Tris-HCl pH 7.5 (50mM)

1 g NH_4Cl

75 mg K_2HPO_4

28 mg $FeSO_4$-$7H_2O$

Ultra-pure water (Milli-Q) to 1L.

Procedure:
1. Prepare plates using 20 mL of agar per plate.
2. Isolates to be tested should be streaked onto the hardened agar.
3. Incubate plates at 37^{o}C for 24 h.
4. If no growth was observed O/N, incubate for another 24 h.
5. At 2 days, subculture each plate to confirm initial observation.

Interpretation
Growth on the medium signifies the ability to utilize the sugar.

APPENDIX D

FAFLP Procedure

DAY 0

Grow strains O/N in 5 mL of the appropriate liquid growth media.

DAY 1 - Isolation of DNA

Isolate genomic DNA via CTAB Isolation:

1. Spin 2 mL liquid culture in a 2 mL centrifuge tube for 2 min at max speed in a microcentrifuge. Discard supernatant, resuspend pellet in another 2 mL liquid culture, repeat the centrifugation, and discard the supernatant. Spin a final time for 30 sec to remove as much liquid as possible.

2. Resuspend pellet in 567 µL TE buffer, then add 30 µL 10% SDS and 3 µL 20mg/mL proteinase K (final concentration is 100 µg/mL proteinase K in 0.5% SDS) to lyse the cells. After mixing thoroughly, incubate for 1.5 h at 37^{o}C.

3. Add 100 µL 5M NaCl warmed to 65^{o}C, mix thoroughly.

4. Add 80 µL CTAB/NaCl solution warmed to 65^{o}C, mix and incubate for 10 min at 65^{o}C.

5. Add 0.75 mL chloroform/isoamyl alcohol, mix, and spin for 10 min at max speed in a microcentrifuge.

6. Remove a total of 600 µL (200 µL three times to minimize loss of pellet) supernatant into a fresh microcentrifuge tube, add 3 µL RNaseA and incubate for 1 h at 37^{o}C.

7. Add 600 µL 25:24:1 phenol/chloroform/isoamyl alcohol, mix, and spin at max speed for 10 min.

8. Transfer another 600 µL (200 µL three times) supernatant to a fresh tube, add 390 µL (0.6 volumes) isopropanol to precipitate the DNA. Do not vortex.

9. Spin DNA for 5 min at max speed, discard supernatant, tap rim of tube on a kimwipe to blot.

10. Resuspend with 100 µL 70% ethanol to wash DNA, vortex, respin DNA for 5 min. Blot excess ethanol on kimwipe as described above.

11. Dry DNA in lyophilizer for 1.5 h; redissolve in 100 µL TE Buffer.

Check quality/quantity of DNA isolated via gel electrophoresis and DNA spec. DNA stocks should be stored frozen at -20^{o}C.

DAY 2 – Preparation for PCR Amplification

Make AFLP templates via digestion of ~1 µg genomic DNA using ApaI and TaqI.

Ligate double strand adapters (ordered as oligonucleotides), reaction mix concentrations as follows:
(Janssen et al. 1996, Vos et al. 1995)
10 µL volume, added to restriction digest consisting of:
0.04 µM *Apa*I adaptor
0.4 µM *Taq*I adaptor
1 U T4 DNA-ligase
1mM ATP in 10 mM Tris-HAc pH 7.5
10 mM MgAc
50 mM KAc
5 mM DTT
50 ng/µL BSA
Incubate reaction mixture for 3 h at 37°C.
Post-ligation mixture diluted to 500 µL with 10 mM Tris-HCl, 0.1 mM EDTA pH 8.0, store at -20°C.

DAY 3 – PCR Amplification of Fragments (resulting in 35-400 bp bands)

An additional preselective amplification step is possible (PCR once with unlabeled primers) before the selective amplification; however, it was found that a one-step selective PCR amplification resulted in more bands with equal intensity to the two step amplification.

PCR Mix:
1.5 µL digested template fragments
0.5 µL A01-6FAM primer
0.5 µL T01-6FAM primer
7.5 µL Amplification Core Mix

PCR steps:

1. Denature at 94°C for 2 min
2. 10x cycle of:
   a) Denature at 94°C for 20 sec
   b) Anneal at [66-(n-1)] °C for 30 sec, where n = the cycle number
   c) Extension at 72°C for 2 min
3. 20x cycle of
   a) Denature at 94°C for 20 sec
   b) Anneal at 56°C for 30 sec
   c) Extension at 72°C for 2 min
4. Final extension at 60°C for 30 min

APPENDIX E

MODIFIED HAWTHORNE LAB PROTOCOL: AFLP SAMPLES ON THE 3730

Prepare Run File

- Enter sample names into file
- Save as text tab delimited

Prepare samples for the 3730

- Prepare HiDi + TAMRA: 776µL HiDi + 24µL each of GeneScan 500 & 2500
- Aliquot 6µL HiDi + TAMRA into notched plate
- Add 4µL PCR product, mixing up and down
- Spin down to remove bubbles
- Instant incubate on the thermocycler: 95 for 2-3 minutes, then place directly on ice

Put sample tray in 3730

- Place the sample plate in the black tray and fit the white lid over the unit. Make sure the tab clicks and the wells are visible from the top.
- Check to be sure the red light is NOT on. Open the right-side door to the 3730 by pulling it out, slide out the rack, and open the door to the stacker furthest away from the machine (or closest to you when pulling it out). Place sample plate in the stacker with the notched corner on the back right. Close the stacker door and then the ABI.

Set up and start your run

At the computer closest to the 3730, select "Plate Manager", Import, and look the shared folder on "dfgil41 H:"

- Copy the file from the flash drive (find in "My Computer") to "Shared Data" folder, "UploadFolder", "Joseph Lab"
- Open folder "Joseph"
- Select the file, then click OK
- Select "Run Scheduler", Search, then type in your file name
- Select your file, hit Add, then press Done
- Press the green triangle to start the run. If the machine is already running, it will automatically start the next run. The plate on the bottom of the stack is the next to be run.
- Fill out the Run Log

APPENDIX F

Abridged Hawthorne Lab GeneMapper Protocol for Analysis

Modified from 05/21/10 version written by Julie Byrd Hebert

General Notes:

If you are using more than one dye: the analysis method in GeneMapper will lock you into co-loading the same primer combinations each time. Keep this in mind when running PCRs and loading the sequencer.

General definitions:

- **Kit** – a set of panels with a set of bin definitions, also called "chemistry kit".
- **Panel** – within a kit, a specific set of analysis options that can be used on a set of samples.
- **Marker** – a specific dye (marker) associated with a given panel.
- **Bin** – an area in which a peak is defined.
- **Peak Size** – the actual size of the peak (in base pairs (bp)), typically on the x-axis.
- **Peak Height** – the fluorescence of the peak, typically on the y-axis.

1. Creating Project and Adding Samples

- Double-click on GeneMapper on the Desktop (Note: may take a while to open)
    - Username: gm
    - Password: josephlab
- If not already set, choose 'AFLP default' in the "Table setting:" pull-down menu

→ File → Add Samples to Project (or test tube button on toolbar)

Note: you may want to change this table setting once you get used to using the program.

- Find and select folder for desired sequencer run, or specific samples (if not using all samples in one folder)
- Click 'Add to List'
- When finished, click 'Add'
- Save Project as: [date]_[primers]_[samples/plate id], or another appropriate name that you can remember. (Ex: 090608_Eact-Paag_plate1) **or** open project if you have one already created to which you need to add samples

2. Setting up Panel

- **Go to Tools → Panel Manager, click on 'Panel Manager' in the left-hand frame menu, then → File → New Kit

- **Name your new kit [initials]_AFLP or something similarly simple and appropriately descriptive. **Only need to do this once, you can it for any number of panels within a single kit.**
- **Choose 'Microsatellite' as type (there is no AFLP designation)
- Click on your new kit in the left-hand frame menu → File → New Panel
- In the right window, you will be able to name your new panel
- Name this panel AFLP_[sample descriptor] or something similarly simple Ex: AFLP_towhees.
- Click on your new panel folder in the left-hand frame menu (this may take a while) → File → New Marker
- Create a new marker for each labeled primer run. Change the names to the dye color (e.g. blue)
- make sure the dye colors are denoted correctly, change size range 0-999, and change marker repeat unit to 9, leave stutter ratio on 0.0
- Click Apply and then OK.

3. Setting up the Analysis Method
- Go to Tools → GeneMapper Manager → 'Analysis Methods' tab. **The following starred steps only need to be performed once.**
- **Click on 'New' to set up a new method
- **Choose 'AFLP' as your analysis type
- **Name your method 'AFLP_[initials]', give it a description if you like, and enter ABI 3730 in the 'Instrument' box.
- Go to the 'Allele' tab
  - Panel: select 'Generate panel using samples'
  - Make sure 'delete' common alleles' is not toggled on if you want to keep monomorphic peaks.
  - Under 'Analyze Dyes', only choose blue (unless you are using more than one).
  - Under 'Analysis Range' choose one above the first two bands in the ladder and one below the two top bands.
- Go to the 'Peak Detector' tab
  - Under 'Peak Detection Algorithm', select 'Advanced'.
  - Under 'Sizing' select 'Partial Sizes'. Change the minimum and maximum to match the numbers in the Analysis Range (above).
  - Under 'Smoothing' select 'Light'.
  - Under 'Peak Detection' check that B = 5000, R = 75.
- Click 'Okay', then click 'Done'.

4. Changing Analysis methods
- Under the Analysis method column in the Samples table

    - Click on the first cell in this column.
    - Select 'AFLP_[initials]' or whatever you named your method.
    - Highlight the whole column by clicking on the top column → fill using Ctrl-D
- Under the Panel column
    - Leave as 'none' (you are creating a panel with this analysis step).
- Size Standard
    - Select the appropriate size standard. If this does not exist, you will need to create your own marker standard.
        - Click on 'New Size Standard'.
        - Choose 'Advanced'.
        - Give the size standard a name (such as the names above) and any description needed to identify the size standard.
        - Make sure the dye color is correct for the size standard.
        - Starting with the lowest number, enter each size standard peak into a row.
        - **IMPORTANT**: YOU MAY NEED TO DELETE THE PEAKS ABOVE 700 IF THE ABI IS NOT COLLECTING DATA BEYOND THAT POINT. OTHERWISE, YOUR MARKER WILL MISMATCH AND YOU CAN'T ANALYZE YOUR DATA.
    - Highlight the whole column by clicking at the top of the column → fill using Ctrl-D.

5. Analysis of Samples

- Click on the "play button" (the green arrow in the toolbar), all samples should run through analysis (NOTE: this will take time, especially when it auto-saves.)
    - All of the symbols in the OS and SQ columns should be green.
- If some SQ's are red:
    - Select the sample(s) and click on the Size Match editor in the toolbar (the button with the red peaks) *Helpful hint*: you might want to make the window bigger by clicking on the corner and dragging it out.
    - → Edit → Override all SQ. This will automatically accept all of the current peaks (which is a lot easier than clicking the override button for each sample separately).
    - **Correct any mislabeled peaks (left click a peak to select, right click to alter) for each sample, then go to the next sample.
    - Once you have checked all of these samples, click 'Ok'.
    - If the size standard is too low for peaks to be called (<25 fluorescence units), note for re-run, and then delete the sample from the project .
    - Note: if this is done, the sample(s) will have to be reanalyzed .
- If some OS's are yellow cautions:

    - Note in the display plots which peaks are off-scale – those peaks will not have accurate size calls, and may throw off the size calls of peaks near them. If those size bins look useful when editing marker bins, you will have to dilute and re-run those samples with off scale reads.
- Once all have been corrected, hit play.
- Double check the quality of your samples in the sample plot window (multicolored peak button on the toolbar)
    - Highlight the entire project; click the sample plot button.
    - Toggle on any/all appropriate colors and scroll through your samples to make sure there are none under-amplified and no samples that look very different than all the others. If there are, note those samples.
- Close the sample plot window.
- If there were any samples that should be deleted, click on the sample then select
  → Edit → Delete from Project.
- Once all have been corrected, hit play. You will need to make sure no "play" symbols are present before you export your project panel.

6. Exporting Project Panel and Bins

→ File → Export Project Panel

- Chemistry Kit: (use your kit name plus an additional character, e.g. JBH_AFLP1)
- Panel Name: (use your panel name plus an additional character, e.g. AFLP_Phytomyza1) If you haven't named your panel yet, put whatever you are planning to label your panel in here.

- Save to Desktop (or an appropriate folder).

7. Editing Project Panel Bins

- On Desktop, two files will have been created
    - Delete the file without 'bin' in the name (panel file).
    - Open the .txt file that does have 'bin' in the name.
- In the text file, change:
    - Kit name: [your kit name] (e.g. SEK_AFLP).
    - BinSet name: AFLP_[names]_BinSet_[whatever you name it].
        - Important: the first two parts don't matter as much, but you must have the "_BinSet_ ".
    - Panel name: [your panel name] (e.g. AFLP_towhees)
- Save and exit Text Editor

8. Importing Bins

→ Tools → Panel Manager

-Highlight your chemistry Kit (e.g JBH_AFLP)
→ File → Import Bin Set

- If you already have a bin set by that name, you will need to either rename your new bin set, **or** delete the current bin set before adding your new one.
    - To delete the current bin set, make sure it is selected in the dropdown window, → Bins → Delete Bin Set.
- Select the text file that was just edited from the desktop and import
- If there are overlapping bins, you will get an error message. The error log can be located at this pathname C:/AppliedBiosystems/GeneMapper/PanelImportlog.txt
    - Open it and note what size bins overlap.
    - Open your bin .txt file on the desktop and change one of the overlapping bin sizes by 0.01bp so they no longer overlap.
    - Save and exit Text Editor.
    - Repeat your import process, it should work.
- Your new binset will now be in the 'BinSet' pull-down menu in the Panel Manager
- Click apply and OK

9. Sample Re-Analysis

- Return to GeneMapper window (samples table)
- Go to Analysis → Analysis Method Editor → Allele Tab
    - Panel: toggle to 'use specified panel'.
    - Bin Set: select the new bin set in the pull-down menu.
    - Click 'OK'.
- Select the column 'Panel'
    - Change 'None' to [your panel name].
    - Select entire column, fill with Ctrl-D.
- Click green arrow to reanalyze the data
- Again, correct any error in size standard (SQ), and if you do, run the analysis again.
    - (See note about correcting size standards under 5. Analysis of Samples.)

10. Editing Marker Bins

→ Tools → Panel Manager

- Highlight your panel in the left-hand frame menu.
- Double check and make sure your bin set is selected in the drop-down menu at the top of the page.
  → Bins → Add Reference Data (or the test tube button on the toolbar)

- Navigate to the samples you just imported and analyzed in this project; they should appear in the bottom left-hand corner of the 'Add Reference Data' window.
    - If you forget to return to the GeneMapper window and RE-ANALYZE after importing your binset, your samples will not be available as reference data.
- **Choose the appropriate folders/samples and click 'Add To List'.
- When finished, click 'Add' (be patient, this sometimes takes a while).
- Your samples will now appear as references samples in the button left-hand frame menu when your PANEL or MARKER is selected.
- Select your blue marker in the upper left-hand frame menu .
- Select your first reference sample in the lower lower left-hand frame menu.
- Deselect all colors other than blue (or the color you are scoring) by toggling the color icons on the toolbar.
- Select all of your reference samples in the lower left-hand frame menu: select the first sample using the mouse, then select the last sample in the list while holding down the 'shift' key (this may take a while, GeneMapper needs to 'think').
- Right click the x-axis and choose 'scale to . . .'
- Select min value = 89 and max value = 91 (Depending on your size standard, you can start scoring at whatever size is greater than the smallest two size markers. I suggest a window of 10-20 base pairs so you can view each peak easily.)
- Right click the y-axis and choose 'scale to maximum'. If you prefer, you can click 'scale to…' and use some useful number (e.g. 1000).
    - Your bins will appear as grey vertical lines.
- Left click to select the marker bin and right click to edit
    - You can also just left click on the bin, then use the left mouse button to drag the bins to make them wider, smaller, or move their position.
- Scan your bins for computer mistakes – are there any unscored 'humps' under 100 fl. units underneath nice, large peaks? Are there any ugly shoulder peaks or overlapping peaks that might interfere with a nice, 1bp size range on the bin? Are there any flat, ugly peaks?
- If the marker bin appears legitimate, right click to rename with the primer combination number and size in bp [e.g. Eact-Pact_119].
    - (I don't usually do this. I just leave the number.)
- Scroll through the entire size range in 10-20 bp windows and repeat the assessment of marker bins – you may have to zoom out on the y-axis to view any large or off-scale peaks.
    - One nice feature is the little red crosses at the top: they represent peaks that were scored as 1's. You can use them to narrow your bins, making it less likely you will get peaks from negative controls.

- Stop scoring at a size where weaker samples start to lose what otherwise appear to be monomorphic peaks (probably 900-950bp in size). If your samples look nice all the way up to the cut-off of the run, stop scoring before you reach the second to largest size standard marker
- Toggle on any colors that were run concurrently and check for pull-up under high peaks.
- Click Apply and OK to exit out of Panel Manager.
- Rerun the analysis, including overriding the ladders. If you did not edit or add any bins, continue on to Step 11.
- If you did change or add any bins, return to the 79-91 bp window and double check those bins.
    - Again, for the bins you changed, you should be able to narrow the bins close to the red crosses.
- Do this for the entire size range.
- Click 'Apply'
- Repeat the scoring process for your green marker
- Export your marker bins for record/reference
    - Click on the chemistry Kit, → File → Export Bin Set
    - Click 'Apply' and OK

11. Sample Re-Analysis

- Return to GeneMapper window (samples table)
- Make sure you have selected the correct analysis, size std. and panel
- Click green arrow to reanalyze the data.
- Select only your negative controls and view them using the view button in the tool bar. (It has a blue, red, and green peak on it.)
- Zoom in by dragging the mouse over a smaller section of one of the samples.
    - If any of the negative controls have a peak, select the button on the top left that shows a bin around a peak. (The one with grey under the peak is the default.)
    - Right click on the bin with the peak in the negative control, and delete the bin.
- Continue checking all of the negative controls by scrolling up and down between the samples, and left and right along the peak size.
- When you close the window, you will get a warning asking if you want to save the Project Panel. Choose Yes.
- Correct any error in size standard (SQ). If you were not correcting your ladders during the process above, make sure to do it now.
- Click on the 'Genotypes' Tab (next to the 'Sample' Tab).
    - Your Genotypes table will now be filled with 0 and 1 scores for all your validated bin sets.

- If not, go to the Table Setting Editor (the button that looks like a table) → Genotypes → Check the Allele button in the scroll down list. Click okay, then the alleles with 0 and 1 scores should show up in your genotype table. You can also just change the Table Setting to "AFLP Default."

→ File → Export Table or Ctrl-E

- Save the table in your folder on the desktop.
- Unless you have the new version of Microsoft office or you are using some shareware with a good spreadsheet, you will likely not be able to view all of the columns in the exported file. Therefore, you may want to make a Table Setting with only the 'Allele" button checked but not peak height, etc.
- Keep a separate excel file of all samples and primer combinations.
- Make sure your samples are always in the same order when pasting new primer combo results into this file.
- Keep backups of everything.

12. Finalized Marker Bins

- When you have run enough samples to encompass the majority of variability (either a predetermined number, or whenever you and the PI make the call), you will not have to create a panel from your samples each time you add new ones to the project.
- Instead, skip straight to the last '11. Sample Re-Analysis' step .
- Once you are satisfied with your marker bins, you may also want to create an Analysis Method for that pair of primer combinations alone so you don't have to constantly edit the AFLP_panel method. Make sure 'user specified panel' is toggled and the appropriate binset is selected.

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