Competence Induction and Transformation-Negative Mutants in *Aeromonas*

by

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ABSTRACT

Members of the genus *Aeromonas* can be isolated from many environmental niches where bacteria exist, but are ubiquitously found in aquatic environments and can be isolated from rivers, lakes, ponds, estuaries, and groundwater. The presence of *Aeromonas* in water systems, especially in drinking water, is an issue of concern in public health. One reason that members of genus *Aeromonas* are a threat to human health is their ability to multiply in drinking water, and this adaptation may be due to their ability to undergo transformation. Transformation is one method of bacterial gene transfer in which extracellular ("naked") DNA is taken up from the environment, and it is entirely dependent on the competence of the recipient cell. Competence is the physiological state that the cell must enter before it can take up extracellular DNA. Competence in *Aeromonas* can be induced under laboratory conditions. The nutrient strength and the content of culture media can affect competence induction in *Aeromonas*. Competence induction is higher in media which have lower nutrient concentrations than in full-strength media. Several factors, such as the pH of the medium and molecules secreted by a growing culture, putative quorum-sensing molecules, can also have an effect on competence induction in *Aeromonas.* This research found that, the competence of *Aeromonas* was highest in the range of pH 6 to pH 8. Media with a pH below 6 or above 9 produce reduced or low numbers of transformants. The putative quorum-sensing molecules negatively affected competence induction in *Aeromonas.* Four putative transformation-deficient strains were isolated by random insertion mutagenesis of strain C-70 and tested for their ability to transform using qualitative and quantitative

transformation assays. There were none or greatly reduced numbers of transformants in all four of the strains.

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CHAPTER 1

INTRODUCTION

1.1. Introduction

This thesis will discuss an overview of the genus *Aeromonas* and will briefly describe *Aeromonas* in the aquatic environment, its adaptability to different environments, its occurrence in drinking water, and its involvement in the health of both humans and other animals such as fish. Transformation and competence induction in *Aeromonas* will be discussed in detail.

1.2. Overview of the genus *Aeromonas*

The species of the bacterial genus *Aeromonas* consist of Gram-negative, facultatively anaerobic rods. *Aeromonas* is oxidase positive, which means that it produces cytochrome *c* oxidases and can therefore utilize oxygen for energy production with an electron transfer chain. *Aeromonas* species are widely distributed in the aquatic environment, including untreated and processed drinking water (Holmes et al., 1996). Aeromonads can be isolated from many environmental niches where bacteria exist. These include aquatic habitats, fish, foods, domesticated pets, invertebrate species, birds, ticks and insects, and natural soils (Janda and Abbott, 2010). Even though *Aeromonas* is mainly found in aquatic environments, it is also found in many other habitats. Neyts et al. (2000) examined different types of foods for the presence of mesophilic *Aeromonas* species both qualitatively and quantitatively, and aeromonads were isolated from vegetable samples, meat and poultry samples, and fish and shrimp. Previously

Aeromonas was mainly known as a fish pathogen, but now it is known that some *Aeromonas* species cause gastroenteritis in humans, mostly in young children and immunocompromised adults. The number of the species of the genus *Aeromonas* has grown to 24 (Janda and Abbott, 2010) within its own family (*Aeromonadaceae*), but only five species (*A. hydrophila, A. caviae*, *A. veronii, A. jandaei,* and *A. schubertii*) are currently recognized as human pathogens (Janda and Abbott, 1998). A number of water- and food-borne cases of diarrhea associated with *Aeromonas* have been reported (Altwegg et al., 1991; Hänninen et al., 1997).

Most aeromonads were viewed in the 1970's as belonging to one of two major groups. The first group is the mesophiles, those strains that grow best at 35ºC to 37 ºC. These *Aeromonas* strains are responsible for a variety of human infections and were commonly referred to as *Aeromonas hydrophila* (Janda and Abbott, 1998). The mesophilic aeromonad species have been commonly isolated from patients with gastroenteritis, although their role in disease causation is not clear (Khan, 2009). The second group is the psychrophilic strains that grow best at lower temperatures (22ºC to 28ºC) and primarily cause infections in fish, such as salmonids. These were designated *Aeromonas salmonicida* (Janda and Abbott, 1998).

Within the past 15 years, the number of named genomospecies in the genus *Aeromonas* has increased from 14 to 24 (Huys et al., 1996; Neyts et al., 2000; Janda and Abbott, 2010). *Aeromonas hydrophila, A. caviae* and *A. veronii* biovar *sobria* are the three genomospecies which are considered most pathogenic for humans (Neyts et al., 2000; Altwegg et al., 1990). *Aeromonas caviae*, *A. hydrophila*, and *A. veronii* biovar

sobria in that order are the *Aeromonas* species most frequently associated with gastroenteritis (Joseph, 1996).

1.2.1. *Aeromonas* in the aquatic environment

Aeromonas is ubiquitiously found in aquatic environments and can be isolated in various concentrations from rivers, lakes, ponds, brackish water (estuaries), drinking water, groundwater, wastewater, and sewage in various stages of treatment (Janda and Abbott, 2010). In view of the increased evidence supporting the role of aeromonads in human diseases, Fiorentini et al. (1998) investigated the occurrence, concentration and diversity of different *Aeromonas* phenospecies and their correlation with growth in two different water bodies. Based on this study, *A. caviae* was the most prevalent species in water with a high degree of pollution, whereas *A. hydrophila* strains were more commonly isolated from cleaner water. *Aeromonas sobria* and *A. veronii* were equally distributed in both polluted and cleaner estuaries. Generally, estuaries which receive untreated waste water are richer in nutrients and subsequently account for higher aeromonad concentrations than those that receive treated waste water. Even though an elevated number of aeromonads might be attributed to a high level of available nutrients, the genus *Aeromonas* is capable of adapting to low-nutrient environments (Fiorentini et al., 1998).

1.2.2. *Aeromonas* in drinking water

The presence of *Aeromonas* in water systems, especially in drinking water, is an issue of concern in public health. In most developing countries, the source water for consumption is not provided though pumps; hence people use untreated water from rivers

which may contain pathogenic bacteria for drinking. Those bacteria can be a source of various diseases and even death. The ability of bacteria to cause disease largely depends on the number of microorganisms, virulence, and ability to survive and adapt to the environment (Gołas et al., 2002). One reason that some bacterial genera, such as *Pseudomonas* or *Aeromonas*, are a threat to human health is their ability to multiply in drinking water (Havelaar et al., 1990).

1.2.3. *Aeromonas* as a pathogen

Aeromonas was not a widely known pathogen several decades ago but these days it is gaining a higher profile. There are several species of *Aeromonas* that are well known as fish pathogens. Fish are susceptible to a wide variety of bacterial pathogens. Furunculosis is one of the most devastating fish diseases to aquaculture. Of the four subspecies of *A. salmonicida* (*salmonicida, achromogenes, masoucida* and *smithia*), subsp. *salmonicida* induces typical furunculosis and causes severe septicemia with resultant mortality especially within cold-water fishes (Cipriano and Bullock, 2001). The other three subspecies of *A. salmonicida* produce the so-called atypical forms of disease that are often characterized by dermal ulcerations and external pathology with or without subsequent septicemia. Even though both typical and atypical subspecies may not be common to all geographic areas, the number, diversity and distribution of fish species that are susceptible to *A. salmonicida* enhance this bacterium's distribution worldwide (Cipriano and Bullock, 2001).

Aeromonads were first recognized only as causing systemic illnesses in poikilothermic (cold-blooded) animals, but now they are known not only as an important

disease-causing pathogen of fish and other cold-blooded species but also as the etiologic agent responsible for a variety of infectious complications in both immunocompetent and immunocompromised persons **(**Janda and Abbott, 2010). *Aeromonas* species are also associated with sepsis and wounds, and with eye, respiratory tract, and other systemic infections (Janda and Abbott, 1996).

1.2.4. *Aeromonas* as a human pathogen

Studies have indicated that three *Aeromonas* genomospecies (*A. hydrophila*, *A. caviae*, and *A. veronii* bv. *sobria*) are responsible for the vast majority of human infections and clinical isolations attributed to this genus. However, isolation alone does not prove pathogenicity, and many of these isolations probably reflect transient colonization (Janda and Abbott, 1998).

Mesophilic members of the genus *Aeromonas* have been associated with a variety of human infections. A major review on the association of *Aeromonas* with human disease has recently been published (Janda and Abbott, 2010).

The virulence factors of these bacteria can include toxin production, biofilm formation, and antibiotic resistance. One reason for increased prevalence of antibiotic resistance is that the cells may acquire DNA containing antibiotic-resistance genes from other cells or from the environment (Kaznowski and Wlodarczak, 1991; Schmidt et al., 2001) which can make treatment more difficult (Ko et al., 1996; Warren et al., 2004). Since *Aeromonas* species are implicated in both human and fish disease, it is important to understand the underlying mechanisms responsible for gene transfer among these organisms.

1.3. Natural transformation in *Aeromonas*

There are three ways that bacterial genetic transfer can happen: transduction, conjugation and transformation. One method of horizontal gene transfer is called transduction. This process involves the transfer of DNA from one cell to another via a replicating virus.

The second method of gene transfer in prokaryotes is called conjugation. Unlike transduction, the donor cell is not killed during this type of gene transfer. In this process, a donor bacterium sends one DNA strand to another bacterium, the recipient, and each of them make it double stranded. This transfer is made by direct cell-to-cell contact or by a bridge-like connection between two bacterial cells.

The third method, transformation, is the process in which a recipient cell takes up naked DNA from the environment, such as DNA released from a dead organism. In order for transformation to happen, the recipient cell must be competent. Competence is the physiological state in which a bacterial cell is able to take up DNA. This can happen either naturally, where certain bacteria are capable of taking up DNA without special treatments, or artificially by making the cell competent using chemicals like $CaCl_2$. The latter is induced by laboratory procedures in which cells are passively made permeable to DNA, using conditions that do not normally occur in nature.

Natural genetic transformation of bacteria encompasses the active uptake by a cell of free (extracellular) plasmid and chromosomal DNA and the heritable incorporation of its genetic information. It is a mechanism of horizontal gene transfer that depends on the function of several genes located on the bacterial chromosome. The term “natural genetic

transformation" (or natural transformation) has been coined to distinguish it from (artificial) in vitro procedures used to introduce DNA molecules into bacterial cells (Stewart and Carlson, 1986). Bacteria are the only organisms capable of natural transformation. It can be considered the genuine bacterial gene transfer process since other gene transfer processes are determined by genes located on plasmids and transposons (conjugation) and on bacteriophages (transduction).

Natural transformation in the genus *Aeromonas* has recently been characterized in our laboratory. Four genes essential for natural transformation in aeromonads have already been identified—*tapY1* (type IV pili biogenesis protein), *ptsI* (phosphoenolpyruvate phosphotransferase protein I), *recBC* (helicase/exonuclease involved in recombination), and *clpA/clpS* (caseinolytic protease) (Huddleston, 2008).

The major goal of this project is to identify additional genes involved in natural transformation of *Aeromonas*, which will help in understanding of the conditions that induce competence and transformation.

Transformation is well studied in the Gram-positive bacterium *Bacillus subtilis*. DNA taken up by Gram-positive bacteria only has to cross one membrane. However, Gram-negative bacteria have cytoplasmic and outer membranes, and in order for transformation to happen, the DNA must pass through both layers. *Aeromonas* can be a transformation model system in Gram-negative bacteria. Previous studies have described competence, natural transformation, and the optimal physiological parameters whereby transformation occurs in *Aeromonas* (Huddleston, 2008). It is also of interest to use

transformation to develop a genetic system to allow researchers to better study *Aeromonas*.

1.4. Research goals

The major goals of this research are:

1. To determine the effect of culture medium composition on competence induction in *Aeromonas* strain C-70.

2. To identify which component of 20% nutrient broth (NB) is the competence inducer. It was previously determined that *Aeromonas* was most competent in 20% NB.

3. To ascertain if pH has an effect on competence in 20% NB

4. To establish the effect of putative quorum-sensing molecules on competence induction.

5. To isolate additional transformation-negative mutants and identify genes that are involved in natural transformation of *Aeromonas.*

1.5. Research hypotheses

1. Nutrient-rich media decrease competence induction in *Aeromonas*.

2. Highly acidic and highly basic media reduce competence induction and natural transformation in *Aeromonas*.

3. The presence of some molecules, such as quorum sensing molecules, positively affects competence induction in *Aeromonas*.

Competence induction and natural transformation in *Aeromonas* can be affected by different factors. The components and proportion of media used to induce competence can be one factor. Other factors can include the presence of some cations and the pH of the media. Different culture media [20% NB, 100% tryptone broth, 20% tryptone broth, 100% glycerol minimal broth (GMB), 20% GMB, 100% casamino acids, 20% casamino acids, 100% peptone broth, and 20% peptone broth] will be used to induce competence in an *Aeromonas* strain. The media will be prepared in full strength and at 20% strength. Quick transformation assays based on the protocol of Huddleston (2008) will be performed. The transformation frequency will be determined to compare which media make the cells more competent. 20% NB was previously determined as the medium which makes *Aeromonas* strains highly competent. This research will also examine the effect of the acidity and basicity of this medium on competence. The pH of 20% NB will be adjusted and the cells grown to induce competence. The number of transformants will be compared to see at which pH the cells are most competent.

Bacteria use quorum sensing to coordinate certain behaviors based on the local density of the bacterial populations. Quorum-sensing molecules and other molecules are found in the supernatant of a microcentrifuged overnight culture. The effect of those molecules on competence induction of *Aeromonas* will be determined.

Identification of the genes involved in natural transformation will be done by randomly mutagenizing an *Aeromonas* isolate with a plasposon, introduced by using the

procedure of triparental mating to obtain gentamycin-resistant chromosomal insertions. After isolating the gentamycin-resistant clones, screening will be conducted in order to identify novel transformation-negative mutants. Genomic DNA will be extracted from these mutants, and the extracted DNA will be digested with Sal I, self-ligated with T4 DNA ligase, and electroporated into *Escherichia coli* JM 109. The electroporated plasmid will be extracted and the DNA sequenced in order to identify the genes involved in the transformation.

CHAPTER 2

MATERIALS AND METHODS

2.1. The effect of culture media on competence induction

Strain C-70 is a derivative of *Aeromonas* strain # 92, subsequently identified by *gyrB* sequence analysis as *A. salmonicida* subsp. *pectinolytica* (J. Huddleston, manuscript in preparation), that was mutagenized with diethyl sulfate to histidine auxotrophy. The eight culture media used were 20% nutrient broth (NB), 100% and 20% tryptone broth, 100% and 20% peptone broth, 100% and 20% casamino acids, and 100% and 20% glycerol minimal broth (GMB). 20% NB contains 1.6 g of Nutrient Broth (EMD Chemicals) and 1 g of NaCl (EM Science) in 1 liter of distilled water; 100% tryptone broth contains 8 g of Bacto Tryptone (Becton, Dickinson and Company; BD) and 5 g of NaCl in 1 liter of distilled water; 100% peptone broth contains 8 g of Bacto Peptone (BD) and 5 g of NaCl in 1 liter of distilled water; 100% casamino acids contains 8 g of Bacto Casamino Acids (BD) and 5 g of NaCl in 1 liter of distilled water; and 100% GMB contains 20 ml of 50X NCE (no carbon E) buffer per liter, supplemented after autoclaving with 1 m*M* $MgSO_4 \cdot 7H_2O$ (Spectrum Chemical) and 0.25% glycerol (Fisher Scientific). 50X NCE buffer contains 197 g of KH_2PO_4, 248 g of K_2HPO_4 (anhydrous), and 175 g of $Na(NH_4)HPO_4 \cdot 4H_2O$ per liter of distilled water. All of the chemicals in 50X NCE buffer were supplied by Fisher Scientific.

Transformation was measured using quantitative transformation assays as described by Huddleston (2008). Competent cells were prepared by inoculating a single colony into

the different media and incubating overnight with aeration at 150 rpm and at 30ºC. Each culture was diluted 1:100 in the same corresponding medium and incubated again with aeration for 24 ± 2 h at 30ºC. The quantitative transformation assays were performed in 1.5-ml microcentrifuge tubes that contained 100 µl of standard transformation buffer, competent cells, and purified *Aeromonas* genomic DNA. The transformation buffer consists of 36.7 m*M* Tris HCl (Sigma) and 16.8 m*M* Tris base (Sigma), pH 7.9, 20 m*M* $MgSO_4 \cdot 7H_2O$, and 50 m*M* NaCl. Competent cells from each different medium used were added in a volume of 40 µl (4.0×10^7 cells). Purified *Aeromonas* genomic DNA was extracted using the AquaPure Genomic DNA Isolation Kit (Bio-Rad Laboratories). Ten µl of this DNA at a concentration 100 ng μl^{-1}were added giving final concentration of 6.7 ng μl^{-1}. The assay mixture was incubated at 30ºC for 60 min. After the initial incubation, transformation was stopped by adding 10 µl of 200 µg ml^{-1} deoxyribonuclease (DNase) I (Sigma) solution, and then it was further incubated at 30ºC for 1 h to degrade free DNA. Controls were performed by adding DNase I to the assay mixture before the initial 60-min incubation to eliminate all transformation. DNA-only and cell-only assays were also performed as negative controls and incubated 48 h at 30ºC. Transformation mixtures were inoculated onto NCE glucose minimal agar. NCE glucose minimal agar contains 20 ml of 50X NCE buffer, 1 m*M* $MgSO_4 \cdot 7H_2O$, 0.25% D-glucose (Sigma) and 15 g of Difco granulated agar (BD) in 1 liter of distilled water. Colonies that grew on NCE glucose minimal agar were considered to be transformants and were counted. To compare the different cultures, transformation frequencies were determined by dividing the number of transformants by the initial number of colony-forming units added [$(4 \pm 1.5) \times 10^7$ CFU per 40 µl].

2.2 The effect of pH on competence induction

Culture media at pH 5 and pH 6 were prepared by adding a few drops of 0.1 *N* HCl into the 20% NB and measured using a pH meter. Similarly, culture media at pH 7, pH 8, and pH 9 were adjusted by adding using 0.1 *N* NaOH.

Strain C-70 was inoculated into 20% NB at all the different pH values and incubated overnight at 30ºC. Each culture was diluted 1:100 in the corresponding medium and incubated again at 30ºC for 24 h. The transformation assay was performed as described in Section 2.1.

2.3 The effect of putative quorum-sensing molecules on competence induction

A single colony of strain C-70 was inoculated into 20% NB and incubated overnight at 30ºC. One and one-half ml of an overnight culture was microcentrifuged in an Eppendorf 5418 centrifuge. A single colony of C-70 was inoculated into a 50:50 mixture of 1 ml of the original culture supernatant and 1 ml of fresh 20% NB. As a positive control, a single colony of C-70 was inoculated into 2 ml of 20% NB. Both tubes were incubated overnight at 30ºC. These overnight cultures were diluted 1:100 in the corresponding medium and incubated again for 24 h. The transformation assay was performed as described in Section 2.1.

2.4 Mutagenesis of *Aeromonas* isolate

Strain # 92 was previously characterized for transformation in our laboratory. The donor *E. coli* GM2163 and the helper *E. coli* HB101 with the plasmid pRK2013 were used in the mutagenesis of *Aeromonas* by triparental mating. Triparental mating is a form

of bacterial conjugation where a conjugative plasmid present in one bacterial strain (the helper) assists the transfer of a mobilizable plasmid present in a second bacterial strain (the donor) into a third bacterial strain (the recipient). Plasposon mutagenesis according to the protocol of Dennis and Zylstra (1998) was performed using triparental matings in order to obtain random insertion mutants. The plasposon (pTn*Mod*-OGm) was propagated in the donor strain *E. coli* GM2163 (*dcm6 dam13*::Tn*9)* in order to obtain unmethylated DNA that would not be degraded by the *Aeromonas* strain upon entry into the cells. The donor strain, recipient *Aeromonas* strain, and *E. coli* HB101 with the helper plasmid pRK2013 were grown in Luria broth which consists of 10 g of Bacto Tryptone, 5 g of Bacto Yeast Extract (BD), and 5 g of NaCl in 1 liter of distilled H_2O overnight. The next day 500 µl of each strain were combined in a 1.5-ml microcentrifuge tube. The combined cells along with the three controls (the donor, helper and C-70) were microcentrifuged at 14,000 rpm for five min at room temperature. The pelleted cells were suspended in 100 µl of NCE buffer and microcentrifuged again for 5 min at room temperature. The pellets were re-suspended in 50 µl of NCE buffer and placed in a small pool on a Luria agar plate. Luria agar consists of 10 g of Bacto Tryptone, 5 g of Bacto Yeast Extract, 5 g of NaCl, and 15 g of Difco granulated agar in 1 liter of distilled water. The mating plate was incubated overnight at 30°C, and the cells were washed off the plate with 3 ml of NCE buffer; 100 µl of the cells were plated onto Luria agar with 30 µg/ml of gentamycin (Fisher Scientific) and 30 µg/ml of ampicillin (Fisher Scientific) and incubated overnight at 30°C to recover mutagenized transconjugant *Aeromonas* cells.

2.5 Screening for transformation-negative mutants

The gentamycin-resistant transconjugants were patch-plated onto 20% Difco nutrient agar (NA) in order to induce competence and then incubated at 30°C for 48 h. The 20% NA consists of 4 g of Nutrient Agar (EMD Chemicals) and 12.6 g of Difco granulated agar in 1 liter of distilled water. Crude DNA was extracted from the prototrophic donor as described by Juni (1974). A small amount of bacterial cell paste on a bacteriological loop, from growth on any suitably plated medium, was carefully placed into 0.5 ml of a lysing solution. The lysing solution consists of sterile 0.05% sodium dodecyl sulfate (Mallinckrodt) in standard saline citrate solution [0.15 *M* sodium chloride and 0.015 *M* Na_3citrate (Fisher Scientific)] contained in a 13 x 100 mm screw-capped tube. The cells were suspended uniformly with the aid of a vortex mixer (Fisher), and the suspended cells were then heated in a 60°C water bath for 15 to 60 min, a procedure which sterilizes the contents of the tube by causing cell lysis and the release of intracellular DNA. Next 400 µl of the crude DNA were spread onto a plate of NCE glucose minimal agar. The colonies on the 20% NA were then replica-printed to the NCE glucose plate with DNA and incubated at 30°C for 48 h. Colonies that did not produce transformants on NCE glucose were tested for the following phenotypic characteristics.

(1) Growth with another carbon source. NCE glycerol agar was made by mixing 15 g of Difco granulated agar in 500 ml of distilled water and 20 ml of 50X NCE buffer in 480 ml of distilled water, which was autoclaved independently, and then adding 1 ml of 1 *M* $MgSO_4 \cdot 7H_2O$ and 0.25% glycerol. This test was performed to make sure the colonies are not general carbon source-utilization mutants because glucose-utilization

mutants and transformation-negative mutants give the same phenotype in the quick assay for transformation.

(2) Adequate growth in NCE glucose supplemented with histidine.

(3) Transformation using quantitative transformation assays as described by Huddleston (2008).

Competent cells were prepared by inoculating a single colony into 20% NB and incubating overnight with aeration at 30ºC. The culture was diluted 1:100 in 20% NB and incubated again for 24 ± 2 h with aeration at 30ºC. Quantitative transformation assays were performed in 1.5-ml microcentrifuge tubes that contained 100 µl of standard transformation buffer. Competent cells were added in a volume of 40 µl (4.0×10^7 cells). Pure *Aeromonas* genomic DNA was prepared according to the method of Ulrich and Hughes (2001) as described in Section 2.6. Ten µl of DNA, in a concentration of 100 ng/µl, were added to the mixture assay. The transformation assay was performed as described in Section 2.1 with a few modifications. The assay mixture was incubated at 30ºC for 30 min. Assay mixtures were diluted in 0.85% NaCl, spread onto NCE glucose minimal agar, and incubated 48 h at 30ºC. Colonies that grew on NCE glucose minimal agar were considered to be transformants and were counted. The transformation frequencies were determined as described in Section 2.1.

2.6 Preparation of genomic DNA

Genomic DNA from the *Aeromonas* culture was extracted according to the protocols of Ulrich and Hughes (2001). A 1.5-ml overnight culture was microcentrifuged at 14,000 rpm (16,873 × *g*) at 4ºC for 2 min. The pellet was resuspended in 500 µL of 50

m*M* EDTA, and then 30 µl of a 100 mg/ml solution of lysozyme (MP Biomedicals) were added. The suspension was incubated at 37ºC for 30-60 min, and then the suspension was microcentrifuged again at 4ºC for 1 min. The pellet was resuspended in 850 µl of Nuclei Lysis Solution (Promega), and three freeze-thaw cycles were performed using a -60ºC freezer and an 80ºC water bath (4-6 min in the freezer followed by 1-2 min in the water bath). Once the lysate had cleared, 4 µl (4 mg/ml) of ribonuclease (RNase) A (Sigma) were added to the mixture, which was incubated for 30 min at 37ºC. Two hundred µl of Protein Precipitation Solution (Promega) were then added to the mixture and vortexed. The lysate was microcentrifuged at 4ºC for 10 min, and the supernatant was transferred to a clean 1.5-ml microcentrifuge tube. The supernatant was microcentrifuged again for another 10 min to remove residual protein. Six hundred µl of isopropanol at room temperature were added to precipitate DNA and incubated at -20ºC overnight. The suspension was microcentrifuged at 4ºC for 8-10 min and washed twice with 70% ethanol. Finally, DNA was resuspended in 20-50 µl of 10 m*M* Tris HCl and quantified using a NanoDrop, ND-1000 spectrophotometer (Thermo Scientific).

2.7 Digestion and self ligation

The purified genomic DNA was digested by the standard protocols. Two µl of DNA (700 ng/µl), 1 µl of Sal I (Promega), 5 µl of 10X Buffer D (Promega), 0.5 µl of bovine serum albumin (BSA) and 41.5 µL of distilled water were combined in a 1.5-ml microcentrifuge tube and incubated in a water bath at 37ºC for 2 h, then heated at 65ºC for 20 min to deactivate the enzyme and washed to completely remove Sal I from the mixture. The enzyme was removed by adding 5 µl of 3 *M* sodium acetate, mixed by

vortexing, adding 110 µl of absolute ethanol and mixed by vortexing again, and then incubated overnight at -20ºC. The mixture was microcentrifuged for 30 min at 13,000 rpm, and then 100 µl of 70% ethanol was added and microcentrifuged for 5 min. The ethanol was removed carefully with a pipette, and the genomic DNA pellet was dried for 10 min at room temperature. The no-enzyme control was treated in the same way but without Sal I. The digested DNA was self-ligated with T4 DNA ligase (Promega) by the following protocol. Ten µL from the restriction digestion were taken for the ligation reaction which also contained 1 µL of T4 DNA ligase (Promega), 5 µL of ligase buffer (Promega), and 34 µl of distilled water. This ligation mixture was then incubated overnight at 16ºC.

2.8 Preparation of electrocompetent cells

Escherichia coli JM 109 was inoculated into 2 ml of Luria broth and grown overnight at 37ºC with aeration. Five hundred µl from the overnight culture were inoculated into 250 ml of Luria broth and grown with aeration at 37ºC until it reached A_{550} = 0.5-0.6 (approximately 3 h). Two hundred ml of this culture were centrifuged in a Sorvall RC-5C refrigerated centrifuge (Thermo Scientific) at 6,000 rpm at 4ºC for 15 min. The cells were resuspended in 200 ml of sterile cold distilled H_2O, and the cells were pelleted again at 6,000 rpm at 4ºC for 15 min.The pelleted cells were resuspended in 100 ml of sterile cold distilled H_2O, and then the cells were pelleted and resuspended in 0.4 ml of 10% glycerol. Finally, the cells were pelleted and resuspended in 250 µl of sterile 10% glycerol for a final 800-fold concentration of cells, and the aliquots were stored in -80ºC.

The ligated DNA fragments from Section 2.7 were electroporated into the electrocompetent cells. The cells, DNA fragments, and cuvettes were kept in ice at all times for best results. The electroporated cells were transferred to 1 ml of SOC medium (Invitrogen) immediately after electroporation, incubated for 1 h with aeration at 37ºC, and then plated onto 20% NA plates containing 30 µg/ml gentamycin.

CHAPTER 3

RESULTS

The degree of competence of the cells depended on the composition and strength of the media. Several media were tested for competence induction in *Aeromonas* strain C-70; as is shown in Table 1, 20% NB had the greatest ability to induce competence and hence gave the highest number of transformants of all media tested. When the cells were grown in 100% tryptone broth, 100% glycerol minimal broth (GMB), 20% GMB, 100% casamino acids, 20% casamino acids, and 100% peptone broth there were no detectable transformants; however, the 20% peptone broth and the 20% tryptone broth had some transformants (Table 1).

The acidity of the media had an effect on the competence of the cells in 20% NB. The 20% NB was adjusted to pH 5, pH 6, pH 7, pH 8, and pH 9. There were no detectable transformants at pH 5 and pH 9. The cells were competent at pH 6 and pH 7, but there was a 5 fold of transformants at pH 8 (Table 2).

Bacterial metabolites presumed to be quorum-sensing molecules were found in the supernatant of an overnight culture. The *Aeromonas* cells grown in 2 ml of 20% NB and the cells grown in a mixture of 1 ml of 20% NB and 1 ml of a supernatant from an overnight culture of the same *Aeromonas* strain were compared for their ability to induce competence on *Aeromonas.* Even though the viable count of the cells grown with the supernatant was reduced by threefold from the cells grown on the 20% NB, which was

most probably due to the prior depletion of nutrients in the supernatant, the number of transformants were more greatly reduced, about fifteenfold (Table 3).

Gentamycin-resistant transconjugants were found from the triparental mating and were patch-plated onto 20% nutrient agar (NA) in order to induce competence and then incubated at 30°C for 48 h. Four hundred µl of the crude DNA were spread onto a plate of NCE minimal medium supplemented with 0.25% glucose. The colonies on the 20% NA were then replica-printed to an NCE glucose minimal plate with DNA and incubated at 30°C for 48 h. Even though most of the colonies produced His^+ transformants, four that did not produce His^+ transformants were found and were considered putative transformation-negative mutants. These four colonies were labeled temporarily as A, B, C, and D, and pure cultures were stored at -80ºC.

These putative transformation-negative mutants were tested for growth in a culture medium containing another carbon source, NCE glycerol minimal medium, to make sure they were not carbon source-utilization mutants because glucose-utilization mutants and transformation-negative mutants give the same phenotype in the quick assay for transformation. These putative transformation-negative mutants did not grow on the glycerol minimal plates but did grow on the glucose minimal plates supplemented with histidine, which showed that they are not glucose-utilization mutants but are most likely transformation-negative mutants. The transformation frequencies of these candidate transformation-negative mutants were determined. There were no detectable transformants from three of them but a reduced number from the fourth, strain B (Figure 1). To identify the genes involved, genomic DNA was extracted from all four strains and

digested with Sal I. As shown in Figure 3, the restriction enzyme digested the chromosomal DNA. The control can be seen as thicker, undigested band, which indicates that the restriction enzyme has cut the chromosomal DNA. The cut genomic DNA was self-ligated with T4 DNA ligase. These self-ligated DNA fragments were electroporated into an *E. coli* host strain. During the electroporation, a positive control with a known plasmid, pUC 19, was also performed. Transformants were recovered from the positive control but not from among the ligated DNA molecules.

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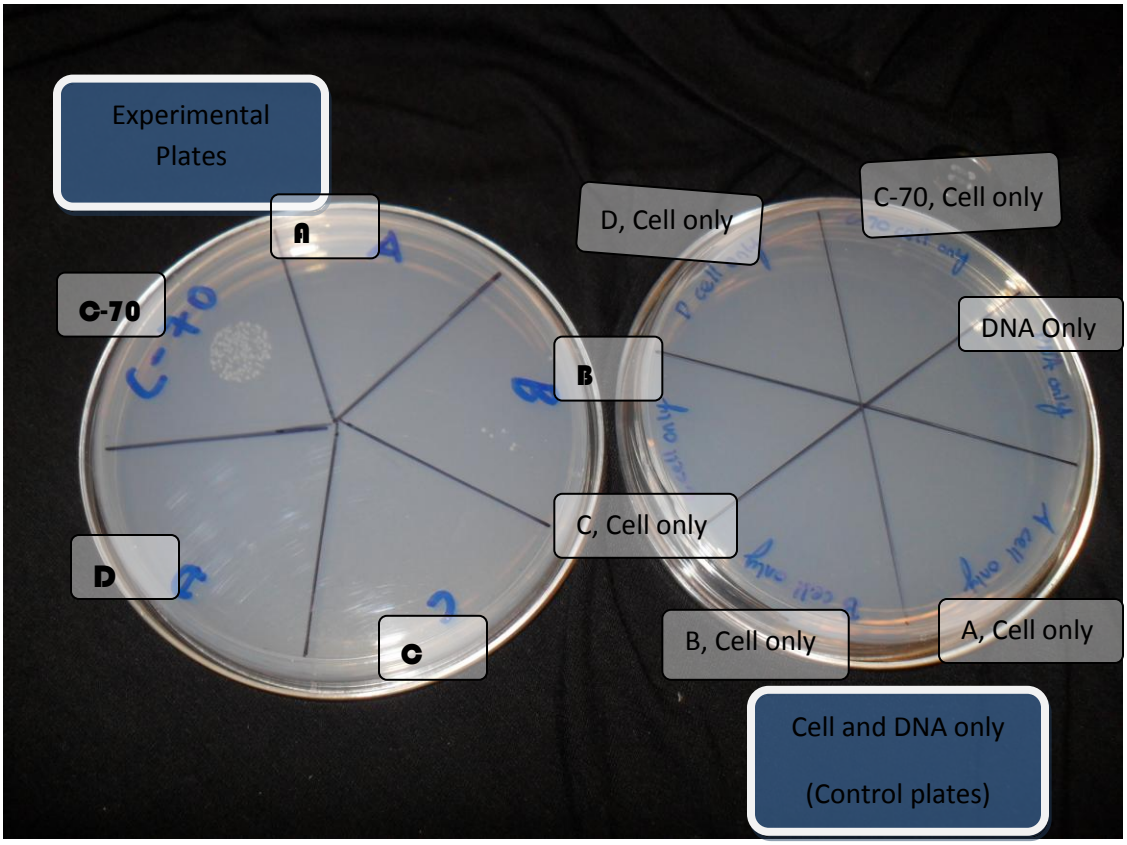

Figure 1: Semi-quantitative transformation assay on NCE glucose minimal plates.

The plate on the right shows all the negative controls (the cells from the different putative transformation-deficient strains without DNA, and DNA only without cells), with no growth in all cases. The plate on the left has the experimental strains (A, B, C, and D) with no detectable or a greatly reduced number of transformants and the positive control strain C-70 with many detectable transformants.

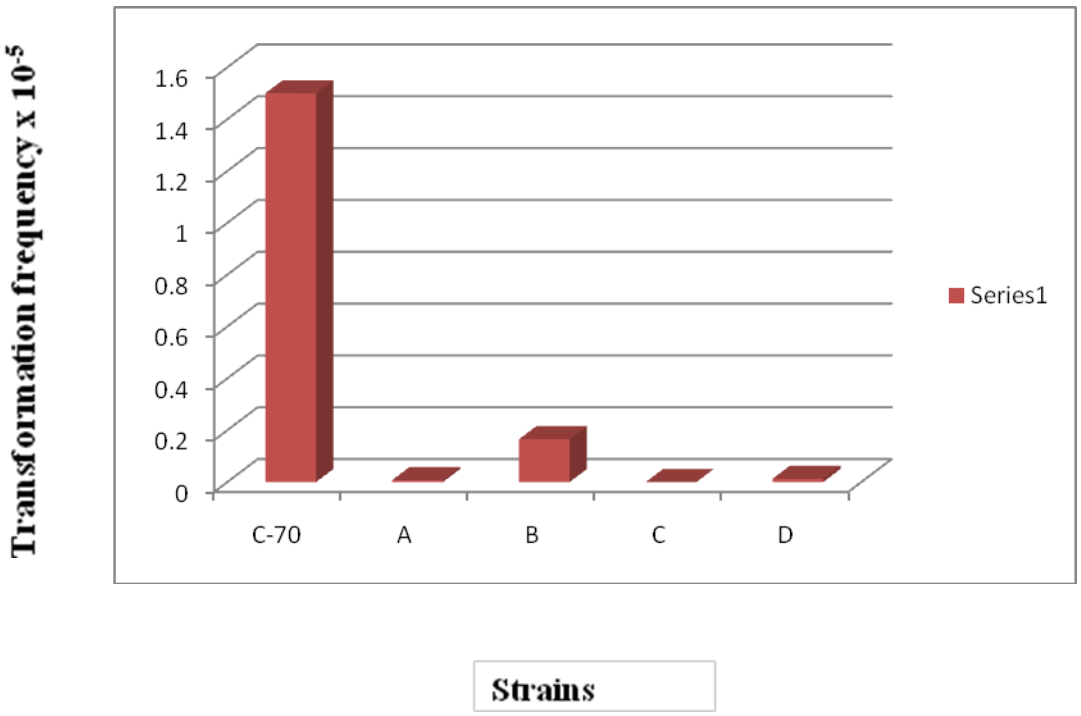

Figure 2: Average Transformation frequencies in *Aeromonas* strains.

The transformation frequencies of the putative mutant strains (A, B, C, and D) and the positive control (C-70). Transformation frequencies were determined by dividing the average number of transformants (600, 3, 66, 0, 5) for the strains C-70, A, B, C, and D, respectively, by the initial number of cells added per assay (4.0×10^7 cells).

Table 1: Effect of culture medium composition on transformation frequency.

Media	Transformants (recipient cell)-1
20% NB broth	1.5×10^{-4}
100% tryptone broth	0
20% tryptone broth	2.75×10^{-6}
100% GMB broth	0
20% GMB broth	0
100% casamino acids	0
20% casamino acids	0
100% peptone broth	0
20% peptone broth	5.25×10^{-5}

The above table shows that *Aeromonas* strain C-70 grown in 20% NB has the highest transformation frequency. The transformation frequencies in 20% tryptone broth and 20% peptone broth are also higher than in the other media tested.

Table 2: Effect of the pH on competence induction of *Aeromonas*.

	pH 5	pH 6	pH 7	pH 8	pH 9
No. of transformants[a]	0	1.41×10^{3}	1×10^{3}	1.7×10^{2}	3×10^{1}
Transformation frequency[b]	0	3.5×10^{-5}	2.5×10^{-5}	4.25×10^{-6}	7.5×10^{-7}

[a] Average number of transformants (colonies) x dilution factor

[b] Transformation frequency = number of transformants/4×10^{7} cells

Table 3: Effect of putative quorum-sensing molecules on competence induction of *Aeromonas.*

	Fresh medium	50:50 Fresh : Spent medium	Ratio
Viable count[a]	1.31×10^{9}	4.1×10^{8}	3:1
Transformants[b]	6.86×10^{3}	1.42×10^{2}	48:1
Transformation frequency[c]	1.3×10^{-4}	8.6×10^{-6}	15:1

[a] Colony-forming units (cfu/ml)

[b] Average number of transformants (colonies) x dilution factor

[c] Transformation frequency = number of transformants/ initial number of cells added per assay

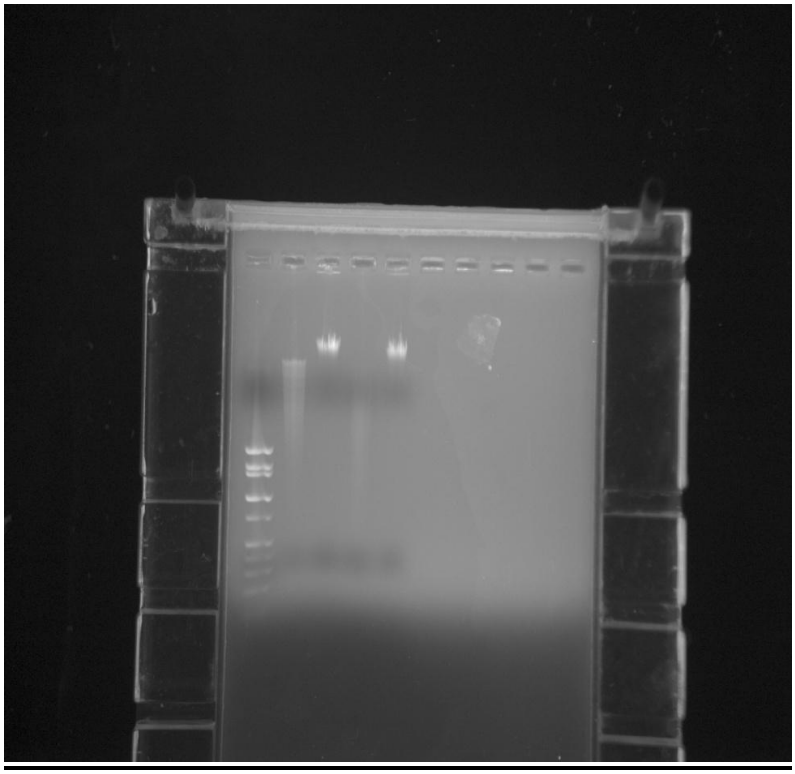

Figure 3: Genomic DNA of strain C, a putative transformation-deficient mutant.

Lane 1: molecular weight markers. Lanes 2 and 4: the DNA digested with Sal I. Lanes 3 and 5: uncut DNA control; the tubes were treated same way as the digestion tubes except that Sal I was not added to the controls. The smear in lanes 2 and 4 shows the digestion goes well.

CHAPTER 4

DISCUSSION AND CONCLUSIONS

Previous research has determined that members of the genus *Aeromonas* have the highest level of competence in 20% NB. As one of my research goals, I tested several different media to find a medium which might induce better competence in *Aeromonas* than 20% NB. Based on the quick transformation assays, there were no detectable transformants on most of the media used to induce transformation. There were some transformants produced in 20% tryptone broth and 20% peptone broth but not as many as in 20% NB, which clearly means that none of the media tested was able to induce competence more effectively than the 20% NB (Table 1). Peptone is one component of nutrient broth. More transformants were observed among the cells grown in 20% peptone broth than the other media tested except for nutrient broth, indicating that one or more chemical components in the peptone may have a positive impact in making the cells more competent.

Once it was determined that *Aeromonas* was most competent in 20% nutrient broth, the research continued to analyze the effect of other factors like the pH of the medium and molecules secreted by a culture during overnight growth in inducing competence in *Aeromonas*. There were no detectable transformants in the medium adjusted to pH 5 but there were high numbers of transformants at pH 6 and pH 7. At pH 8 the numbers of transformants were greatly reduced and there were very few transformants at pH 9. These results indicated that the competence of *Aeromonas* is highest in 20% NB between pH 6 and pH 8. Based on these results, it can be concluded

that competence is not induced or is greatly reduced under more acidic and basic pH conditions. Some proteins necessary for competence induction may be affected by the pH of the medium. Extremely low or high pHs than the optimal pH may result in the loss of protein function due to conformational change or may completely degrade it.

The other factor tested for its effect on induction of competence in *Aeromonas* was the molecules remaining in the supernatant after centrifugation of an overnight culture to remove whole cells; i.e., putative quorum-sensing molecules. This possibility was tested using fresh 20% NB as a positive control and a 50:50 mixture of the spent medium plus fresh medium. Since the amount of the nutrient availability in the 50:50 mixture is reduced, viable counts were first determined. The viable counts of the overnight culture grown in the mixture were reduced approximately threefold but the transformants produced in it were reduced by fifteenfold, showing that the putative quorum-sensing molecules may be negatively affecting the competence induction of *Aeromonas*. Further research should be done to exactly identify those molecules and at what concentration they would greatly affect the competence induction and transformation of *Aeromonas*.

Auxotrophs do not grow in minimal media. In order for the histidine (His) auxotrophic strain C-70 to grow in minimal media, the media must either be supplemented with the nutrient required by the auxotroph (His) or the strain should be able to take up DNA from its prototrophic strain (# 92). C-70 is able to take up DNA from the environment by transformation (Huddleston 2008).

Isolating transformation-deficient mutants was performed by random insertion mutagenesis of strain C-70 using conjugation (triparental mating) to introduce the transposable genetic element pTn*Mod*-OGm (plasposon). Mating mixtures were inoculated onto plates containing two antibiotics (ampicillin and gentamycin) to select the *Aeromonas* transconjugants that inherited the chromosomal insertions of the plasposon from the donor *E. coli* GM 2163. The resulting transconjugants were tested for their ability to transform using a quick, qualitative transformation assay as described in Section 2.5, and from several thousands of transconjugants, four strains apparently were unable to take up the DNA in the minimal medium. These four strains remained auxotrophic for histidine and they did not grow when a different source of carbon, glycerol, was used, showing that they are transformation deficient and not glucose-utilization mutants.

Quantitative transformation assays were performed to determine the transformation frequency of these putative transformation-deficient strains and to compare their transformation efficiencies with the parent strain, C-70, which is also a positive control for the experiment. There were none or greatly reduced numbers of transformants in all the four strains. These phenotypic results showed that these strains are indeed transformation deficient.

The question remains: what makes them transformation deficient? In the experiments carried to identify the genes involved, the whole DNA appeared as thick band on a gel showing that the concentration of DNA was sufficient for cloning. The negative control for the digestion was uncut and indicated that the genomic DNA from

the transformation-deficient mutants was cut as expected. The digested DNA was supposed to be self ligated and electroporated into *E. coli* JM109. The positive control for the electroporation produced expected number of transformants. However, transformants were not recovered from among the self-ligated DNA molecules. The ligation mixture and the control without T4 DNA ligase appeared to be similar on the agarose gel, which suggested that the problem of not getting transformants from the electroporation is most probably lack of ligation. Hence this research will continue using additional techniques to favor the self ligation.

REFERENCES

1. **Altwegg, M., L. G. Martinetti, J. Luthy-Hottenstein, and M. Rohrbach.** 1991. *Aeromonas*-associated gastroenteritis after consumption of contaminated shrimp. Eur. J. Clin. Microbiol. Infect. Dis. 10:44-45.
2. **Chen, I., and D. Dubnau.** 2004. DNA uptake during bacterial transformation. Nature Rev. Microbiol. 2:241–249.
3. **Cipriano, R. C., and G. L. Bullock**. 2001. Furunculosis and Other Diseases Caused By *Aeromonas salmonicida.* United States Department of the Interior, Fish and Wildlife Service, Division of Fishery Research, Washington, D. C. 20240, Fish Disease Leaflet 66.
4. **Dennis, J. J., and G. J. Zylstra.** 1998. Plasposons: modular self-cloning minitransposon derivatives for rapid genetic analysis of Gram-negative bacteria genomes. Appl. Environ. Microbiol. 64:2710-2715.
5. **Fiorentini, C., E. Barbieri, L. Falzano, P. Matarrese, W. Baffone, A. Pianetti, M. Katouli, I. Kuhn, R. Möllby, F. Bruscolini, A. Casiere, and G. Donelli.** 1998. Occurrence, diversity and pathogenicity of mesophillic Aeromonas in estuarine waters of the Italian coast of the Adriatic Sea. J. Appl. Microbiol. 85:501-511.
6. **Gołaś I., Z. Filipkowska, D. Lewandowska, and I. Zmysłowska.** 2002. Potentially pathogenic bacteria from family *Enterobacteriaceae*, *Pseudomonas* sp. and *Aeromonas* sp. in water designated for drinking and household purposes. Polish J. Environ. Stud. 11:325-330.

7. **Hänninen M. L., P. Oivanen, and V. Hirvelä-Koski.** 1997. *Aeromonas* species in fish, fish-eggs, shrimp and freshwater. Inter. J. Food Microbiol. 34:17-26.
8. **Havelaar A. H., J.F. Versteegh, and M. During.** 1990. The presence of *Aeromonas* in drinking water supplies in the Netherlands. Zentralbl. Hyg. Umweltmed. 190:236-256.
9. **Heinemann, J. A.** 1991. Genetics of gene transfer between species. Trends Genet.7:181–185.
10. **Holmes, P., L. M. Niccolls, and D. P. Sartory.** 1996. The ecology of mesophilic *Aeromonas* in the aquatic environment, p. 127–150. *In* B. Austin, M. Altwegg, P. J. Gosling, and S. Joseph (ed.), the genus *Aeromonas*. John Wiley & Sons Ltd., West Sussex, England.
11. **Huddleston, J. R.** 2008. Natural transformation in *Aeromonas* species. Ph.D. Dissertation, Texas Tech University.
12. **Huys, G., R. Coopman, P. Janssen, and K. Kersters.** 1996. High resolution genotypic analysis of the genus *Aeromonas*. Int. J. Syst. Bacteriol. 46:572-580.
13. **Janda, J. M., and S. L. Abbott.** 1996. Human pathogens, p.151-173. *In* B. Austin, M. Altwegg, P. J. Gosling, and S. Joseph (eds.), the genus *Aeromonas*. John Wiley & Sons Ltd., West Sussex, England.
14. **Janda, J. M., and S. L. Abbott.** 1998. Evolving concepts regarding the genus *Aeromonas*: an expanding panorama of species, disease presentation, and unanswered questions. Clin. Infect. Dis. 27:332–344.
15. **Janda, J. M., and S. L. Abbott.** 2010. The genus *Aeromonas*: taxonomy, pathogenicity, and infection. Clin. Microbiol. Rev. 23:35–73.

16. **Joseph, S. W.** 1996. *Aeromonas* gastrointestinal disease: a case study in causation? p. 311–335. *In* B. Austin, M. Altwegg, P. J. Gosling, and S. Joseph (eds.), the genus *Aeromonas*. John Wiley & Sons Ltd., West Sussex, England.

17. **Juni, E.** 1974. Simple genetic transformation assay for rapid diagnosis of *Moraxella osloensis*. Appl. Microbiol. 27:16-24.

18. **Kaznowski, A., and K. Wlodarczak.** 1991. Susceptibilities of motile *Aeromonas* sp. to antimicrobial agents. Zentralbl. Bakteriol. 275**:**85-93.

19. **Khan M. R.** 2009. Role of TapY1 and PlsB in biofilm formation in *Aeromonas* species. Master's Thesis, Texas Tech University.

20. **Ko, W. C., K. W. Yu, C. Y. Liu, C. T. Huang, H. S. Leu, and Y. C. Chuang.** 1996. Increasing antibiotic resistance in clinical isolates of *Aeromonas* strains in Taiwan. Antimicrob. Agents Chemother. 40:1260-1262.

21. **Lorenz, M. G., and W. Wackernagel.** 1994. Bacterial gene transfer by natural genetic transformation in the environment. Microbiol. Rev.58:563–602.

22. **McMahon, M. A. S., and I. G. Wilson.** 2001. The occurrence of enteric pathogens and *Aeromonas* species in organic vegetables. Int. J. Food Microbiol.70:155–162.

23. **Merino, S., X. Rubires, S. Knochel, and J. Thomas.** 1995. Emerging pathogens: *Aeromonas* spp. Int. J. of Food Microbiol. 28, 157-168.

24. **Miller, S. A., D. D. Dykes, and H. F. Poleskey.** 1988. A simple salting out procedure for extracting DNA from human nucleated cells. Nuc. Acids Res. 16:1215.

25. **Neyts K., G. Huys, M. Uyttendaele, J. Swings, and J. Debevere.** 2000. Incidence and identification of mesophilic *Aeromonas* spp. from retail foods. Lett. Appl. Microbiol. 31:359-363.

26. **Schmidt, A. S., M. S. Bruun, I. Dalsgaard, and J. L. Larsen.** 2001. Incidence, distribution, and spread of tetracycline resistance determinants and integron-associated antibiotic resistance genes among motile aeromonads from a fish farming environment. Appl. Environ. Microbiol. 67:5675-5682.

27. **Stewart, G., and C. Carlson.** 1986. The biology of natural transformation. Annual Review of Microbiol. 40:211–235.

28. **Ulrich, R. L., and T. A. Hughes**. 2001. A rapid procedure for isolating chromosomal DNA from *Lactobacillus* species and other Gram-positive bacteria. Letters in Appl. Microbiol. 32:52-56.

29. **Warren, W. J., R. M. Jeter, R. C. Kimbrough, and J. C. Zak.** 2004. Population patterns and antimicrobial resistance of *Aeromonas* in urban playa lakes. Can. J. Microbiol. 50:397-404.