Natural transformation in *Aeromonas* species

by

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A Dissertation

In

BIOLOGY

Submitted to the Graduate Faculty
of Texas Tech University in
Partial Fulfillment of
the Requirements for
the Degree of

DOCTOR OF PHILOSOPHY DEGREE

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May, 2008

ACKNOWLEDGMENTS

This dissertation and accompanying research would not have been possible without the support and assistance of many people. Dr. Mohamed Fokar at the Texas Tech University Center for Biotechnology and Genomics gave molecular advice. Dr. Gerben Zylstra of the Biotechnology Center for Agriculture and the Environment, Cook College, Rutgers University, New Brunswick, New Jersey, generously provided the plasposons and antibiotic resistance cassettes for the mutagenesis experiments. Jeb Clark, research associate, spent many months amplifying and preparing genes for sequencing so that definitive species identifications of the bacterial strains in this study could be possible. Trisa Crutcher at the Texas Tech University Center for Biotechnology and Genomics sequenced all of the genes. Angela Moss, Mary Catherine Hastert, and Steven Platten of the University Imaging Center of Texas Tech University assisted with electron microscopy. Waters Agricultural Laboratories, Inc., Owensboro, Kentucky, analyzed water samples. Heath Grizzle and Colin Bell gave statistical guidance. Jason Jitjumnong assisted with DES mutagenesis and auxotroph isolations. Texas Tech University Department of Biological Sciences gave financial support in the form of teaching assistantships, research assistantships, and travel grants. TechASM also gave travel grants and other types of financial assistance. The SBC Chancellor's Fellowship provided three years of continuous financial support. Finally, Dr. Randall Jeter, Dr. Clifford Fedler, Dr. Abdul Hamood, Dr. Michael San Francisco, and Dr. John Zak gave much needed research advice and encouragement.

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ABSTRACT

Members of the genus *Aeromonas* are ubiquitous aquatic bacteria that can be causative agents of diseases in fish, amphibians, reptiles and mammals, including humans. Aeromonads are capable of natural genetic transformation, a process whereby genetic information is horizontally transferred between bacterial cells. This study described the optimal physiological parameters whereby transformation occurs. Competence was induced in 20% nutrient broth during the stationary phase of growth. Optimal transformation assay conditions for one chosen isolate were in Tris buffer with magnesium or calcium, pH 5 to 8, and a saturating concentration of 0.5 µg of DNA per assay (3.3 ng DNA μl^{-1}) at 30ºC. Sodium was also required and could not be replaced with ammonium, potassium, or lithium. The maximal transformation frequency observed was 1.95×10^{-3} transformants $(\text{recipient cell})^{-1}$. Transformation was evaluated among environmental aeromonads by assaying auxotrophic recipients with point mutations (n = 46) with donor DNA from other wild-type, prototrophic aeromonads under optimal conditions. The results demonstrated that 57% of the isolates were able to act as recipients, and 100% were able to act as donors to at least some other aeromonads. *Aeromonas* species only took up DNA from closely related species, and not all species became competent under the same conditions. Four genes essential for transformation were identified—*tapY1* (type IV pili biogenesis protein), *ptsI* (phosphoenolpyruvate phosphotransferase protein I), *recBC* (helicase/exonuclease involved in recombination), and *clpA/clpS* (caseinolytic protease). These genes indicate mechanisms of competence development through starvation (*ptsI*) and stress

responses (*clpA/clpS*), DNA binding (*tapYI*), and recombination (*recBC*). To better understand the potential for *Aeromonas* transformation under natural conditions, assays were done using playa water. The water from three Lubbock, Texas lakes—Maxey, Higinbotham, and Stevens—was collected monthly for one year and filter-sterilized prior to assay. Transformation frequencies were analyzed with respect to levels of bicarbonate, boron, calcium, carbonate, chloride, conductivity, magnesium, nitrate nitrogen, pH, phosphorus, potassium, sodium, sulfate, sodium absorption ratio, and total dissolved solids measured in each lake. Transformation frequencies were lower in the water (maximum 1.90×10^{-5}) when compared to optimal transformation buffer, yet still took place, indicating the process could occur in the lakes and serve as a natural environment for gene exchange.

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0/129	vibriostatic compound—2,4-diamino-6,7,-di-iso-propylpterdidine phosphate
°C	degrees Celsius
µg	microgram
µl	micoliter
µm	micrometer
µ*M*	micromolar
ANOVA	analysis of variance
ATCC	American Type Culture Collection
BLAST	Basic Local Alignment Search Tool
cAMP	cyclic adenosine monophosphate
CFU	colony-forming units
clp	caseinolytic protease gene
Crp	cAMP-cAMP receptor protein
DES	diethyl sulfate
df	degrees of freedom
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DUS-R	DNA-uptake-sequence receptor
EI	enzyme I
EIIA	enzyme II
g	g-force
GMB	glucose NCE minimal broth

gyr	DNA gyrase gene
h	hour
Hpr	histidine protein
kbp	kilobase pairs
kDa	kilodalton
L	liter
LB	Luria broth
M	molar
min	minute
ml	milliliter
mm	millimeter
m*M*	millimolar
mmhos/cm	millimhos per centimeter
N	normal
NB	nutrient broth
NCBI	National Center for Biotechnology Information
NCE	no citrate E
ng	nanogram
nm	nanometer
P	probability
PCR	polymerase chain reaction
Pil	pilin protein
ppm	parts per million
ptsI	phosphoenolpyruvate phosphotransferase protein

rec	recombination gene
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
SE	standard error
sec	second
ssDNA	single-stranded DNA
tap	type IV pili gene
Tn	transposon
TSB	tryptic soy broth

CHAPTER I

INTRODUCTION

Overview of the Genus *Aeromonas*

Members of the bacterial genus *Aeromonas* are Gram-negative, facultatively anaerobic rods found ubiquitously in aquatic habitats (66). They are oxidase positive, resistant to vibriostatic agent 0/129, and do not require NaCl for growth (124). There are three different phenotypic groups composed of the type species *Aeromonas hydrophila*, *A. caviae*, and *A. sobria,* while there are at least eighteen different DNA hybridization groups (25). New species are discovered and characterized often. Since 2004, three novel species have been described: *A. molluscorum* (108), *A. simiae* (60), and *A. bivalvium* (109).

Aeromonads can be found in almost every aquatic environment. Higher densities of *Aeromonas* are isolated from water during warmer versus cooler temperatures worldwide (53, 122), including in Lubbock, Texas (68, 171). They are found in both polluted and unpolluted environments as well as in chlorinated drinking water distribution systems around the world (48, 52, 70, 88), both raw and treated sewage (61, 111, 127), and in the oceans at the freshwater/marine interfaces (62). Aeromonads can also be found thriving in deep groundwater (105). They have also been isolated from drinking water (53, 160) and foods, such as poultry (137), vegetables (121), shellfish (108, 109), and fish (179).

Aeromonads have also been found in association with mosquitoes (125), monkeys (60), eels (46), leeches (73), and houseflies (116). Since fish, amphibians, and many reptiles live in aquatic environments, they come into contact with aeromonads. Aeromonads can cause infections in all of these organisms. Furunculosis, which occurs in salmonids, is caused by *A. salmonicida* and is one of the most devastating fish diseases to aquaculture. *Aeromonas hydrophila* can cause redsore disease in bass, as well as tail and fin rot, and hemorrhagic septicemias in other freshwater fish (11). The bacteria also cause red leg disease in frogs (89), fatal infections in turtles (123), and skin lesions and septicemia in crocodiles (163).

Differentiating the species of *Aeromonas* is difficult. There are no reliable identification schemes based on phenotypic characteristics. However, an isolate can usually be identified to one of the three phenotypic groups using a few biochemical tests (1, 69). Controversy exists when speciating aeromonads based on 16S rRNA gene sequences (103, 148). The gene has a high similarity (98-100%) between different species (103). There have also been contradictions between DNA-DNA hybridization data and 16S rRNA gene data. Recently *gyrB* (175), *rpoB* (91), *rpoD* (149), and *dnaJ* (117) have each been used independently to successfully distinguish the different members of the genus *Aeromonas* without contradicting DNA-DNA hybridization data.

It is important to differentiate between the species because members of the genus *Aeromonas* are not only fish and amphibian pathogens but are also considered to be a threat to human health (92). Aeromonads have been implicated in self-limiting

gastrointestinal infections and associated with both childhood diarrhea in developing countries (4, 5, 87, 155) and traveler's diarrhea in Africa, Latin America, and Asia (167).

Although direct evidence for aeromonad causation of diarrhea is limited, the *Aeromonas* genome encodes genes for the enterotoxins Act, Alt, and Ast, and also for hemolysins. Act is a cytotoxic enterotoxin with hemolytic activity that contributes to *Aeromonas* pathogenicity (174). Alt and Ast are cytotonic enterotoxins that, like Act, are unrelated to cholera toxin (4, 96). Together, Act, Alt, and Ast have been shown to evoke diarrhea in mice (144).

In addition to intestinal infections, *Aeromonas* has been linked to hepatobiliary infections, especially in immunocompromised patients (31). Hemolytic uremic syndrome in adults has also been linked to aeromonads in rare instances (49). One healthy child is suspected to have acquired a verocytotoxic *A. sobria* infection that caused renal failure. The aeromonad was presumably obtained from her bathtub in which an aquarium had been previously cleaned (50).

Extraintestinal infections commonly occur when a wound is exposed to *Aeromonas*-contaminated water. The most common *Aeromonas* wound infections in humans are attributed to *A. hydrophila*, *A. veronii*, *A. jandaei*, *A. trota*, and *A. schubertii* (71). Wound infections can progress quickly and may ultimately be fatal if the infection becomes systemic. Cellulitis, myonecrosis, and ecthyma gangrenosum are the results of wound infections and are treated with broad-spectrum and extended spectrum antibiotic therapy, such as cephalosporins and chloramphenicol (180). The latter two types of

infections may progress seriously enough for amputation to be required. People have become infected with *Aeromonas* through incidents involving bear bites (90), tiger bites (45), mud football (164), boating (95), snake bites (75), stingrays (128), motorcycles (22), near-drowning events (110), and burns (82). Many of these wounds were immersed in *Aeromonas*-contaminated water shortly after the accident occurred. The most common skin and soft-tissue infections after the 2004 tsunami in Thailand were due to *Aeromonas* (65). Furthermore, high densities of *Aeromonas* were found in the flood waters of Hurricane Katrina in New Orleans, creating a potentially infectious environment for injured evacuees (129).

Nosocomial infections from *Aeromonas* can also occur. Leeches used medicinally after some surgeries such as replants, grafts, and reconstructive surgery can cause infection due to wound exposure to *A. hydrophila* residing in the digestive tract of the annelid (9, 35, 47, 141). Other types of nosocomial infections that have been reported include necrotizing fasciitis (30), septic arthritis (151), biliary tract infections due to drainage devices (31, 41, 67, 162), and post-operative scar infections (34).

Immunocompromised individuals are susceptible to *Aeromonas* infections. Reported infections in these patients are necrotizing fasciitis (2, 162), meningitis (94), septic arthritis (134), pneumonia (114), diarrhea (135), septicemia (84), and urinary tract infections (67).

Environmental, food, and clinical aeromonad isolates have demonstrated increasing resistance to antimicrobial agents, thereby making treatment of infections

difficult (85, 121, 171). These resistances have been shown to be transferred through plasmids (79, 142) in *Aeromonas*. Members of other bacterial genera such as *Bacillus*, *Neisseria*, *Pseudomonas*, and *Vibrio* are capable of transferring genetic information through natural transformation. This type of horizontal gene transfer in *Aeromonas* is not widely known or studied. In 1987, Sakai (138, 139) demonstrated that *Aeromonas salmonicida* is capable of competence and transformation but did not develop the research further. Sequencing of 16S rRNA genes indicates that horizontal gene transfer probably has occurred over the evolutionary history of the *Aeromonas* genus (112, 148). Aeromonads possess several characteristics that are common in other naturally competent Gram-negative bacteria, such as type IV pili (14), type III secretion system (172), and biofilm formation (83). The recently completed genome sequence of *A. hydrophila* ATCC 7966^T indicates the presence of a competence/damage-inducible protein CinA and a DNA internalization-related competence protein ComEC/Rec2 (143). No links between these components in *Aeromonas* and transformation have been made, and no other research has characterized this process in this genus or considered the ecological or clinical consequences of transformation in this ubiquitous, opportunistic pathogen.

Natural Transformation

Natural genetic transformation is the process whereby bacterial cells take up free DNA from their environment and incorporate it into their genomes. This mode of horizontal gene transfer was first observed by Griffith in 1928 in *Streptococcus pneumoniae* (55).

There are several models explaining possible reasons why bacteria take up DNA from the environment. One model is that this process occurs in order for DNA to be used as a nutrient source (51). This model is supported by studies that show some bacterial strains become competent to take up DNA under nutrient-limited conditions (27, 133). Another model is that transformed DNA molecules serve as templates for DNA repair. Bacterial cells exposed to ultraviolet light have greater survivorship when they are treated with DNA after damage than if they are not. The added DNA presumably serves as a template in recombinational repair (107). Transformation, specifically the *com* regulon, is induced in *S. pneumoniae* upon exposure to mitomycin C, a DNA-damaging agent (132). In addition to nutrition and template acquisition, natural transformation may allow the cell to purposely obtain novel genetic information. However, a recent study on transformability in *Acinetobacter baylyi* by Bacher et al. (13) revealed that competent strains and noncompetent strains adapted to laboratory conditions at the same rate. Competence did not confer any discernable advantage in this study, and the competent lineages even evolved a lower level of transformability under laboratory conditions. Whatever the biological reason for the uptake, one consequence of transformation is a changed genotype of the recipient cell. Transformation is widespread among microorganisms and has a lasting impact on prokaryotic genomes that is difficult, if not impossible, to assess (17).

The process of natural genetic transformation has been broken down into six major steps (99). They are (a) DNA is released from donor cells, (b) DNA is dispersed, (c) DNA persists in the environment, (d) recipient cells become competent for DNA

uptake, (e) competent cells interact with and take up the DNA, and (f) genes encoded on the donor DNA are expressed in the recipient cells.

Release, dispersal, and persistence of donor DNA in the environment. Free DNA has been found in every environment studied including in sediment, seawater, and freshwater. A large portion of this DNA is bacterial in origin (99). Under laboratory conditions DNA has been found in the culture liquid of both Gram-positive and Gram-negative bacterial isolates from soil. This DNA is excreted into the broth during all growth phases, during cell lysis, and during competence development (98). Some bacteria can regulate when and how much DNA they release. Competent *Streptococcus pneumoniae* has been shown to lyse noncompetent cells resulting in release of DNA (152, 153). Intact bacterial cells also release DNA into the surrounding environment. This was first demonstrated in *Pseudomonas stutzeri.* Living donor cells yield a higher transformation frequency than DNA alone (154).

Active release of DNA into the environment in the absence of cell lysis can occur through two known mechanisms. The first mechanism is DNA transport through membrane-derived vesicles, also referred to as "blebs". Blebs containing DNA have been identified in fourteen different Gram-negative species, such as *Agrobacterium tumefaciens*, *Borrelia burgdorferi, Escherichia coli, Moraxella osloensis, Neisseria gonorrhoeae,* and *Shigella flexneri* (40). These blebs can transfer genes from one bacterial genus to another as in the case of the transfer of virulence genes from *E. coli* O157:H7 to *Salmonella enterica* (176). The second type of active release of DNA occurs through the secretion of DNA not enclosed in vesicles. Secretion of DNA from intact

cells was proposed to have occurred because of observed high concentrations of DNA in laboratory cultures of *Neisseria meningitidis* (26), *Bacillus subtilis* (159), and *Pseudomonas aeruginosa* (59). This DNA could transform recipients. A mechanism for this DNA release has been identified in *N. gonorrhoeae* through a type IV secretion system (58).

After DNA is released from the donor cells, the DNA must be dispersed and persist in the environment. Deoxyribonucleases (DNases) are ubiquitous in the environment, but a fraction of the DNA is not degraded. DNA that binds to quartz, feldspar, and clay minerals is protected from destruction by DNases. In addition, DNA adsorbed to sand, silt, and clay is resistant to DNase I degradation and capable of retaining its transforming abilities (136). DNA adsorbs to the surface of sand particles, aiding in increased transformation frequency in aeromonads (138). DNA persists in the soil and has the potential to serve as a genetic reservoir (126). In streams and other aqueous environments, DNA is disseminated by water currents, allowing genome fragments to be dispersed far from the donor cell (93).

Under laboratory transformation assay conditions, it has been shown for both *P. stutzeri* and *Actinobacillus actinomycetemcomitans* that 1.0 μg ml^{-1} (equivalent to 1.0 mg L^{-1}) is saturating (24, 168). DNA in concentrations as low as 0.2 μg L^{-1} is also able to transform recipient cells (168). In estuarine water samples, concentrations of DNA have been found up to 44.0 μg L^{-1} in estuarine water, 25.6 μg L^{-1} in eutrophic fresh water, and 3.2 μg L^{-1} in oligotrophic freshwater (99).

Biofilms could also provide a source of free DNA in the natural environment. Baur et al. (16) postulate that if only one cell in a biofilm were to lyse, this would release a high enough concentration of DNA to neighboring cells that natural transformation could occur. This DNA concentration could conceivably exceed the laboratory saturating concentration of 1 μg ml^{-1}. Williams et al. (173) showed that *Acinetobacter calcoaceticus* in biofilms of are capable of transformation on stones in rivers under natural conditions. DNA can also form a stable extracellular filamentous network (20), though it is unknown if DNA in this network has a role in transformation.

Competence induction. In order for a recipient bacterial cell to be genetically transformed, it first must be in a competent state. The competent state allows cells to bind and take up DNA from the environment or the culture medium. *Neisseria* is the only bacterial genus with members known to be constitutively competent (99). In other transformable species, competence must be induced. During competence development, there is expression of genes that are required for DNA binding, processing, and uptake. Many genera of bacteria have been found to have naturally competent members, including achromobacters (77), *Acinetobacter* (63), *Actinobacillus* (168), *Azotobacter* (119), *Bacillus* (150), *Campylobacter* (170), *Haemophilus* (56), *Moraxella* (76), *Mycobacterium* (118), *Neisseria* (26), *Pseudomonas* (24), *Streptococcus* (55), and *Vibrio* (72). To date, over 90 prokaryotic species have been characterized as naturally transformable. These species are from diverse groups such as photolithotrophs, chemolithotrophs, heterotrophs, methylotrophs, clinical pathogens, and members of *Archaea* (36, 99).

Several mechanisms have been implicated in the development of competence. In *Haemophilus influenzae*, competence is dependent on cAMP-cAMP receptor protein (Crp) complex, which is thought to act as a regulator of competence (27, 100). In order to induce competence in a laboratory setting, *H. influenzae* is grown in rich medium and then transferred to a starvation medium, thus increasing levels of cAMP-Crp complexes (64). This model of competence development in *H. influenzae* supports the hypothesis that DNA uptake could be a means of obtaining nutrients (39, 51). In addition, *B, subtilis* competence is induced by growth in minimal medium supplemented with L-tryptophan (8). In *Vibrio cholerae*, competence is induced by starvation or stress, high bacterial population, and by chitin (15, 106).

Induction of competence in streptococci occurs by means of several other mechanisms. One way is through biofilm formation where a competence-stimulating peptide is released during quorum sensing (157). Competence can also occur in streptococci as a result of increased calcium concentration. Calcium is absolutely required for competence to be induced in this genus (161). *Mycobacterium smegmatis* competence also depends on calcium, whereas magnesium enhances transformation but cannot replace calcium (118). However, in *Azotobacter vinelandii*, magnesium is essential for transformation and calcium cannot substitute (120). The precise roles of these divalent cations in competence and transformation are unknown.

The expression of competence genes in *B. subtilis* is well elucidated. Nutritional, growth-stage-specific, and cell-type-specific regulation of competence all occur in *B. subtilis* (42). The gene *spoOA* is not only involved in the control of sporulation but also

in the control of competence (3). The onset of competence induction occurs at the end of logarithmic phase (8). SpoOA negatively regulates AbrB, which both positively and negatively regulates other genes at several points along the pathway, leading to the expression of the late competence genes (43). There are many different genes that are involved in competence and transformation. Several of these genes and the bacterial genera they have been found in are listed in Table 1.1.

Competence is induced and controlled by a variety of factors, including antibiotics. Aminoglycoside and fluoroquinolone antibiotic exposure in *S. pneumoniae* induces the expression of the *com* regulon as part of a stress response (32, 132). In *Streptococcus*, competence development is also coordinated with bacteriocin production (86). It is proposed that the cells excrete a bacteriocin that kills other closely related cells and that the competent state of the bacteriocin-producing cells allows them to take up the DNA of the recently killed cells.

DNA binding, fragmentation, and uptake. The genes induced during competence encode proteins that are responsible for DNA binding and uptake. After competence induction occurs, the DNA binds to the surface of the recipient cell. In Gram-positive cells, such as *B. subtilis,* DNA binding is non-specific. Non-homologous DNA is subsequently bound and taken up (42). Double-stranded DNA is bound to pilin-like ComG proteins on the outside of the cell (12).

The ComG proteins mediate the binding of the DNA to ComEA found within the cell wall. NucA cleaves the double-stranded DNA, which is then passed through a

cytoplasmic membrane channel formed by ComEC. With the assistance of ComFA, the DNA passes through the channel to the cytoplasm of the bacterial cell (12). The proton gradient is the source of energy for this translocation (101). The DNA is single-stranded when it enters into the cytoplasm, and the non-transported strand is degraded (44).

The mechanism of DNA transport in Gram-negative cells differs from that of Gram-positive cells. In many Gram-negative cells, such as *H. influenzae* (145), *N. gonorrhoeae* (54), and *A. actinomycetemcomitans* (168), only homologous DNA with a species specific uptake signal sequence is taken up, although a specific DNA-binding receptor for these sequences has not yet been identified. These sequences are distributed throughout the genome with a bias towards genome maintenance genes (33). The uptake sequences are also thought to have a dual function as rho-independent transcriptional terminators (7). More research is being done to elucidate this relationship between the two functions of the sequences.

In *P. stutzeri*, maximal competence occurs during early stationary phase where only double-stranded DNA greater than 10 kbp—neither single-stranded nor plasmid DNA—is capable of transforming the recipient cell (24). The DNA uptake system in many Gram-negative cells is similar to the components of type IV pili and type II protein export systems (12, 104, 172). Type IV pili in *P. aeruginosa* have been shown to bind DNA directly (165).

In *N. gonorrhoeae*, double-stranded DNA is recognized by an unidentified DNA-uptake-sequence receptor (DUS-R) and is then transported through the outer membrane

through the PilQ channel. PilP acts as a chaperone for PilQ (12). ComE binds to the double-stranded DNA, and a PilE/ComP complex transports the DNA through the periplasm and the cell wall. Other proteins such as PilM, PilN, and PilO are also necessary for transformation, although their functions and cellular localization remain undetermined (12). ComA transports the DNA through the cytoplasmic membrane with the aid of an unknown translocase. Single-stranded DNA enters the cytoplasm of *N. gonorrhoeae* (12).

Expression of new DNA. Once the donor DNA is taken up from the environment, it can be incorporated into the recipient's genome through homologous recombination. In *B. subtilis*, 50% of the DNA taken up is incorporated into the genome (21). There are several steps in this integration process. First, the single-stranded, cleaved donor DNA is brought into close proximity with the chromosome. Next, an unstable donor-recipient complex is formed, followed by the formation of a stable noncovalent complex. Lastly, the chromosome is repaired and is covalently closed (147). The new DNA can then be expressed.

Transformation in the natural environment. Bacterial transformation in the environment has previously been demonstrated with *Bacillus* (136), *Enterococcus* (102), *Pseudomonas* (37), *Acinetobacter* (98, 173), and even *E. coli* (16). Most of these experiments were performed in sterile soil and/or sterile water microcosms. Sakai (138) demonstrated that *A. salmonicida* is capable of transformation in river sediment, while Sandaa and Enger (140) showed that the same species can serve as a plasmid donor in

marine sediment. Microcosm and in situ experiments are valuable in determining if a particular microorganism is capable of transformation outside the laboratory.

Goals

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. It is also of interest to develop a genetic system to allow researchers to better study *Aeromonas*. The major goal of this research is to demonstrate how natural transformation in members of the *Aeromonas* genus occurs. First, this will be done by describing optimal laboratory conditions for this process. This will facilitate future research by providing a protocol to induce competence and manipulate the genome through transformation. It will also provide some insight as to how *Aeromonas* may undergo transformation in the natural environment. Second, the interspecies and intraspecies transformation capabilities of different isolates of *Aeromonas* will be evaluated. This will demonstrate how widespread competence is in *Aeromonas* and if all species and isolates can be induced to competence under the same conditions. It will also reveal the range of DNA that can be taken up by individual isolates and how closely related DNA donors and recipients need to be in order to have successful transformation occur. Third, several genes necessary for transformation will be identified. Knowing the genes involved will allow understanding of the conditions that induce competence and transformation. Fourth, aeromonad transformation ability will be evaluated in sterile microcosms consisting of playa water to determine if transformation can indeed take place under natural conditions as well as in the laboratory. The results from all of these

aspects of transformation in *Aeromonas* can help researchers to understand the genus more fully and possibly give more information as to how other bacteria may be undergoing transformation at the molecular, ecological, and evolutionary levels.

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Table 1.1. Bacterial genes involved in natural transformation.

Gene	Organism	Function in transformation	Other known functions	Reference
cj0011c	*Campylobacter jejuni*	DNA receptor	DNA binding	74
clpP	*Bacillus subtilis*	Competence	Protein degradation	113
coiA	*Streptococcus pneumoniae*	Recombination	DNA processing	38
comC	*Streptococcus mutans*	Competence	Quorum sensing	6
comE	*Bacillus subtilis*	DNA uptake	DNA binding	131
comEA	*Bacillus subtilis*	DNA uptake	DNA binding	131
comEC	*Bacillus subtilis*	DNA uptake	Membrane permease	28
comF	*Synechocystis* sp.	Competence	Phototactic motility, piliation	115
comFA	*Bacillus subtilis*	DNA uptake	ATPase	97
comGA	*Bacillus subtilis*	DNA uptake	Pseudopilus	57
comGC	*Bacillus subtilis*	DNA uptake	Pseudopilus	29
comGD	*Bacillus subtilis*	DNA uptake	Pseudopilus	29
comGE	*Bacillus subtilis*	DNA uptake	Pseudopilus	29
comGG	*Bacillus subtilis*	DNA uptake	Pseudopilus	29

Table 1.1. Continued.

Gene	Organism	Function in transformation	Other known functions	Reference
comK	*Bacillus subtilis*	Competence	Transcription regulator	19, 166
ctsD	*Campylobacter jejuni*	Competence	Type II secretion protein	172
ctsE/comGA/pilT	*Campylobacter jejuni*	Competence	Type II secretion protein	172
ctsF/pilG/ctsG	*Campylobacter jejuni*	Competence	Type II secretion protein	172
cya	*Haemophilus influenzae*	Competence	Adenylate cyclase, nutrient utilization	39
dprA/smf	*Bacillus subtilis*	DNA uptake	Protects ssDNA	158
dprA+	*Haemophilus influenzae*	DNA uptake	Protects ssDNA	78
dprA	*Helicobacter pylori*	DNA uptake	Protects ssDNA	146
dprA	*Neisseria meningitidis*	DNA uptake	Protects ssDNA	156
dprA	*Streptococcus pneumoniae*	DNA uptake	Protects ssDNA	18
nucA	*Bacillus subtilis*	DNA processing	Endonuclease	130
Pil genes	*Synechocystis* sp.	Competence	Type IV pili	177, 178
pilABCD	*Actinobacillus actinomycetemcomitans*	Competence	Type IV pili	169

Table 1.1. Continued.

Gene	Organism	Function in transformation	Other known functions	Reference
pilN	*Neisseria meningitidis*	Competence	Type IV pili biogenesis	156
pilQ	*Neisseria meningitidis*	DNA binding	Outer membrane channel	10
ptsI	*Neisseria meningitidis*	Unknown	Phosphoenolpyruvate-protein	156
radA	*Streptococcus pneumoniae*	Recombination	DNA damage repair	23
recA	*Bacillus subtilis*	Recombination	ssDNA binding ATPase	81
recBCD	*Acinetobacter baylyi*	Recombination	Helicase/exonuclease complex	80
recG	*Neisseria meningitidis*	Competence	ATP-dependent DNA helicase	156
recN	*Bacillus subtilis*	Recombination	ATPase	81
rho	*Neisseria meningitidis*	Unknown	Transcription termination	156

CHAPTER II

PHYSIOLOGY OF TRANSFORMATION IN *AEROMONAS* SPECIES

Abstract

Aeromonas species are common inhabitants of aquatic environments and relevant as human pathogens. Their potential as pathogens may be related in part to lateral transfer of genes associated with toxin production, biofilm formation, antibiotic resistance, and other virulence determinants. Natural transformation has not been characterized in aeromonads. DNA from wild-type, prototrophic strains that had been isolated from environmental sources was used as donor DNA in transformation assays with auxotrophs as the recipients. Competence was induced in 20% nutrient broth during the stationary phase of growth. Optimal transformation assay conditions for one chosen isolate were in Tris buffer with magnesium or calcium, pH 5 to 8, and a saturating concentration of 0.5 µg of DNA per assay (3.3 ng of DNA μl^{-1}) at 30ºC. Sodium was also required and could not be replaced with ammonium, potassium, or lithium. The maximal transformation frequency observed was 1.95×10^{-3} transformants (recipient cell)$^{-1}$. A survey of *Aeromonas* auxotrophic recipients (n = 46), assayed with donor DNA from other wild-type aeromonads under optimal assay conditions, demonstrated that 57% of the aeromonads were able to act as recipients, and 100% were able to act as donors to at least some other aeromonads. Three different transformation groups were identified based on the isolates' ability to share DNA. The transformation groups roughly correlated to species groups. These results demonstrated that *Aeromonas* environmental

isolates are capable of natural transformation with the DNA from closely related species of *Aeromonas*.

Introduction

Members of the bacterial genus *Aeromonas* are Gram-negative, facultatively anaerobic rods found ubiquitously in aquatic habitats (13). *Aeromonas* isolates have been divided into at least 18 DNA hybridization groups, and each validly named species has a separate hybridization group designation (21). A limited number of biochemical tests can be used to place most strains into one of three phenotypic complexes (1). The *A. hydrophila* complex is comprised of *A. hydrophila*, *A. bestiarum*, and *A. salmonicida*. The *A. caviae* complex is comprised of *A. caviae*, *A. media*, and *A. eucrenophila*. The *A. sobria* complex is comprised of *A. veronii* biogroup sobria, *A. jandaei*, *A. schubertii*, and *A. trota* (1).

Aeromonads are implicated in self-limiting gastrointestinal infections (3, 4, 19) and extraintestinal infections, with wounds serving as the usual portal of entry (15). Both environmental and clinical aeromonad isolates demonstrate resistance to antimicrobial agents, making the treatment of infections more difficult (18, 37). These resistances can be transferred through plasmids (17, 30) or through the process of natural transformation. Approximately 90 prokaryotic species have been characterized as naturally transformable (10). These species are from diverse ecological groups, such as photolithotrophs, chemolithotrophs, heterotrophs, methylotrophs, clinical pathogens, and members of *Archaea* (20).

Natural transformation in *Aeromonas* is not widely known or studied. Sakai (28, 29) reported that a single strain of *A. salmonicida* was capable of competence and transformation but gave no detailed descriptions of the mechanisms involved or the conditions under which transformation occurred. No research has characterized this process in any aeromonad, nor have the evolutionary or clinical consequences of transformation in these ubiquitous aquatic organisms been considered. The goals of this study were to determine if *Aeromonas* species other than *A. salmonicida* are competent for natural transformation, characterize optimal conditions for transformation in the laboratory, determine if all aeromonads can be induced to competence under the same conditions, and assess the scope of transformability within the genus *Aeromonas*.

Materials and Methods

Bacterial strains and identifications with *gyrB* sequences. *Aeromonas* strains used in this investigation were isolated from rivers and playas in west Texas and New Mexico. DNA was extracted and purified from each strain using the method of Ulrich and Hughes (33). The *gyrB* gene sequences using the same primers and conditions as described by Yañez et al. (39) were amplified in order to obtain species identifications of the isolates. The PCR products were treated with ExoSAP-IT (USB Corporation, Cleveland, Oh.) to remove primers and extra dNTPs and sequenced at the Core Facility of the Texas Tech University Center for Biotechnology and Genomics. Sequences were analyzed using Vector NTI, 9.0.0 (Invitrogen, Carlsbad, Ca.) and were compared against all microbe sequences deposited in NCBI using BLAST (5). Three aeromonads from the American Type Culture Collection (ATCC), *A. hydrophila* ATCC 7965, *A. caviae* ATCC

15468^T, *A. veronii* biovar sobria ATCC 9071, were used to confirm the quality of the sequences and identifications.

Mutagenesis and screening of aeromonads. Forty-three wild-type environmental aeromonads and the three *Aeromonas* ATCC strains were mutagenized to auxotrophy with diethyl sulfate (DES) (Sigma-Aldrich, St. Louis, Mo.). Each aeromonad strain was inoculated into 2 ml of no citrate E (NCE) minimal medium (9) supplemented with 0.25% glucose (Sigma) and incubated with aeration overnight at 30ºC. The culture was diluted 1:10 (0.5 ml into 4.5 ml of NCE buffer) in a screw-cap tube; 4 drops of DES were added and incubated at room temperature for 1 h. Then 0.1 ml of the DES-treated cells was inoculated into 2 ml of Difco tryptic soy broth (TSB) (Becton Dickinson, Sparks, Md.). The cells were incubated with aeration at 30ºC overnight, and 0.1 ml was inoculated into 2 ml of NCE glucose minimal medium and incubated for 2 h at 30ºC. Cefuroxime (Sigma) (a cephalosporin), which was stored and prepared according to the manufacturer's instructions, was added for a final concentration of 30 μg ml^{-1} to counterselect for non-growing, auxotrophic cells. The cells were then incubated at 30ºC for 3 h. The culture was diluted in NCE buffer, spread onto Difco tryptic soy agar (TSA) (Becton Dickinson), and incubated overnight. Colonies were patched to TSA, incubated overnight, and replica-plated to NCE glucose minimal agar. Auxotrophs that did not grow on minimal medium were subcultured and used in later experiments.

Quantitative transformation assays. Competent cells were prepared by inoculating a single colony into 20% nutrient broth (NB) (Becton Dickinson), pH 7.5, and incubating with aeration overnight at 30ºC. The culture was diluted 1:100 in 20% NB

and incubated again with aeration for 24 ± 2 h at 30ºC. The competency protocol was derived through trial and error in preliminary experiments. Quantitative transformation assays were performed in 1.5-ml microcentrifuge tubes that contained 100 µl of standard transformation buffer consisting of 36.7 m*M* Tris HCl (Sigma) and 16.8 m*M* Tris base (Sigma), pH 7.9, 20 m*M* $MgSO_4$ (Spectrum Chemical, Gardena, Ca.), and 50 m*M* NaCl (EM Science, Gibbstown, N. J.). The buffer formulation was originally based on modifying 10 m*M* Tris, pH 8.0 through the addition of different combinations of components. Competent cells were added in a volume of 40 µl (4.0×10^7 cells). Pure DNA from each wild-type aeromonad was prepared according to the method of Ulrich and Hughes (33). One µg of DNA was added in a volume of 10 µl (final concentration of 6.7 ng μl^{-1}). The assay mixture was incubated at 30ºC for 30 min. After the initial incubation, transformation was stopped by adding 10 µl of 200 µg ml^{-1} DNase I (Sigma) solution and then incubating at 30ºC for 1 h to degrade free DNA. Controls were performed by adding DNase I to the assay mixture before the initial 30-min incubation to eliminate all transformation. No-DNA and no-cell assays were also performed as negative controls; no transformants ever appeared on any of the control plates. 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. Transformants were counted, and 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].

Determining optimal competence induction medium. One auxotroph (C-70, a histidine mutant derived from putative *A. salmonicida* strain 92) was chosen to characterize optimal competence induction and transformation conditions. A single colony was inoculated into 20% NB and incubated with aeration overnight at 30ºC. The culture was diluted 1:100 in 20%, 40%, 60%, 80%, and 100% NB; 20% and 100% Luria broth (LB); 20% and 100% TSB; and NCE glucose minimal broth supplemented with 0.1 m*M* L-histidine (Sigma) and incubated with aeration overnight at 30ºC. Transformation assays were performed in triplicate in standard transformation buffer as described above. Transformation frequencies were used as an indicator of competence.

Effect of growth phase on competence. One colony of *Aeromonas* C-70 was inoculated into 2 ml of 20% NB, pH 7.5, and incubated with aeration overnight at 30ºC. The 2-ml culture was inoculated into 200 ml of 20% NB and incubated at 30ºC with aeration. Samples were taken hourly for 24 h and then at 36 h and 48 h. The absorbance of each sample was measured at a wavelength of 540 nm using a Spectronic Genesys 5 spectrophotometer (Spectronic Instruments, Rochester, N. Y.). Samples were diluted in 0.85% NaCl and plated onto 20% Difco nutrient agar (Becton Dickinson), pH 7.5, and incubated at 30ºC overnight for viable counts. Samples were also assayed for transformation as described above. This procedure was repeated with three different flasks and two replicates per flask at each sampling time.

Characterization of optimal transformation conditions. Assays for determining optimal transformation conditions were performed as described above except that each parameter was varied independently. Preliminary experiments indicated that

NaCl and $MgSO_4$ are required for transformation to occur. Concentrations of NaCl and $MgSO_4$ were varied to determine optimal concentrations of each to maximize transformation. Varying concentrations of KCl (Fisher Scientific, Pittsburgh, Pa.), LiCl (Sigma), and NH_4Cl (Fisher Scientific) were tested in place of NaCl. Varying concentrations of $CaCl_2$ (Mallinckrodt Baker, Phillipsburg, N. J.) were tested in place of $MgSO_4$. Other variations included Tris concentration, temperature, pH, DNA concentration, and DNA incubation time with cells before adding DNase I. Assays were performed in triplicate or quadruplicate.

Statistical analysis. One-way ANOVAs using Matlab (version 6.0.0.88 Release 12) were used to determine significant effects of assay conditions on transformation frequencies obtained. Frequency data were square-root/arcsine transformed for all analyses to obtain homogeneous analyses. After each ANOVA was performed, Tukey's honestly significant difference criterion was used in a multiple comparison procedure to determine differences among treatments.

Scope of transformability. Quick transformation assays were performed in order to determine the scope of transformability among diverse aeromonad strains isolated from different aquatic sources. Six of the 46 assays were repeated in triplicate. Crude DNA from the wild-type (prototrophic) strain was prepared using the procedure of Juni (16). Briefly, a pellet of each bacterial culture was suspended in lysis buffer and incubated at 70ºC for 1 h, killing cells and inactivating any bacteriophages that might be present, and yielding approximately 1 $\mu g\ \mu l^{-1}$ of crude DNA. Each auxotrophic strain was tested for uptake of DNA from all of the other wild-type strains. Assay buffer (50

µl) was added to 1.5-ml microcentrifuge tubes along with 20 µl of competent aeromonad cells and 10 µl of crude DNA. Each assay was incubated at 30ºC for 1 h, and 20 µl were inoculated as a grid on NCE glucose minimal agar without DNase I treatment. The plates were incubated at 30ºC for 48 h, and numbers of transformants were counted. If assays yielded uncountable colonies, then 50 colonies were used in the statistical analysis. Cluster analysis using the City Block metric was used in Matlab to identify separate transformation groups.

Results

Identification of competent aeromonad. C-70, a histidine auxotrophic mutant, was obtained through DES mutagenesis of a prototrophic strain, *Aeromonas* sp. strain 92, isolated from river sediment from the north fork of the Brazos River (14). This wild-type strain was tentatively identified as a species of *A. salmonicida* based on *gyrB* sequence and is atypical in that it grows at both 30 and 37°C. Preliminary results showed that C-70 is easily transformable and does not spontaneously revert to prototrophy at a detectable frequency [$< 2.50 \times 10^{-8}$ transformants (recipient cell)$^{-1}$]. C-70 was used for optimization of competence induction and transformation conditions.

Competence induction conditions. Competence in *Aeromonas* C-70 is inducible. Cells grown in 20% NB yielded the greatest transformation frequencies. Full-strength standard growth media, both complex and defined, inhibited transformation (Table 2.1). Competence began to appear during stationary phase at about 12 h after inoculation into fresh medium (Figure 2.1) and was maintained through at least 48 h.

Maximal competence occurred in late stationary phase at 22 to 24 h after inoculation. ANOVA results demonstrated that there was a significant difference ($P \leq 0.001$) between transformation frequencies obtained from different phases of growth. The multiple comparison analysis also supported this conclusion. The means of the transformation frequencies from assays taken at hours 22 to 24 did not significantly differ from one another but were significantly higher than assays taken at other time points during growth.

Optimal transformation conditions. Transformation assays without NaCl did not yield any transformants, indicating that sodium is required. Maximal transformation frequencies occurred with 33 m*M* NaCl (Figure 2.2). Other monovalent cations, K^+, Li^+, and NH_4^+, could not replace Na^+ (Figure 2.2) and yielded transformation frequencies that were barely detectable [0 to 3.9×10^{-5} transformants (recipient cell)$^{-1}$]. Transformation was also dependent on either magnesium or calcium. Maximal transformation frequencies occurred with 17 m*M* $MgSO_4$ [8.5×10^{-4} transformants (recipient cell)$^{-1}$] or with 17 m*M* $CaCl_2$ [1.8×10^{-3} transformants (recipient cell)$^{-1}$] (Figure 2.3). Magnesium was added to subsequent transformation assays instead of calcium so as to ensure that natural transformation was occurring as opposed to chemical (artificial) transformation. Tris concentration had less influence on transformation frequency than sodium and divalent cations did (Figure 2.3). Preliminary experiments indicated that higher transformation frequencies occurred in Tris buffer than in phosphate (NCE) buffer. Sterile distilled water with added sodium and magnesium yielded transformants. Strong transformation occurred from 0 to about 50 m*M* Tris, pH 8.0, and decreased with higher

concentrations. The optimal temperature for transformation was 30ºC [$(1.28 \pm 0.12) \times 10^{-3}$ transformants (recipient cell)$^{-1}$] (Figure 2.4). Transformation frequency increased with increasing temperature from 15 to 30ºC and decreased at 37ºC. At 25°C the transformation frequency was $(9.63 \pm 0.80) \times 10^{-4}$ transformants (recipient cell)$^{-1}$. There was a significant difference in the frequencies obtained from transformation assays incubated at different temperatures ($P \leq 0.001$). When the temperature groups were compared pairwise, the means of the frequencies obtained from incubating at 25°C and 37°C were not significantly different from one another, whereas incubating at 30°C significantly increased transformation frequency. Buffer pH had only a small effect on aeromonad transformation in the pH range of 5 to 9. Transformation frequencies varied between 0.97×10^{-3} (at pH 9) to 1.8×10^{-3} (at pH 5) transformants (recipient cell)$^{-1}$ (data not shown). When multiple comparisons were performed, there was no significant difference in the mean frequencies obtained from assays with pH 5, 6, 7, and 8. Frequencies for assays with pH 9 were significantly lower than those from pH 5 to 8. Subsequently, the quantitative assay protocol was always performed at pH 7.9. Transformation frequency was greatest with a DNA concentration of 0.5 µg of DNA per assay and did not increase with DNA concentration up to 4 µg of DNA per assay (Figure 2.5). This conclusion was supported by the multiple comparison analysis. The longer the time (up to 120 min) that DNA was incubated with competent cells before adding DNase I, the greater the transformation frequency [$(3.81 \pm 0.28) \times 10^{-4}$ transformants (recipient cell)$^{-1}$] (Figure 2.6). The results from the ANOVA showed that there was a significant difference between the different DNA incubation times ($P \leq 0.001$). The pairwise multiple comparisons demonstrated that there was a significant difference in the means of

transformation frequencies when incubating the assays for less than 120 min, but frequencies did not significantly increase with incubation times beyond 120 min (Figure 2.6). There was an approximate 10-fold variability between the competence of C-70 between experiments performed on different days. The means of the transformation controls using the optimal conditions for the quantitative transformation assays varied from 0.138×10^{-3} to 1.33×10^{-3} transformants (recipient cell)$^{-1}$ (n = 20). The reversion frequency of the histidine mutant back to prototrophy was less than 2.50×10^{-8} transformants (recipient cell)$^{-1}$, since no revertants were detected in any of the no-DNA control assays.

Scope of transformability. Forty-six aeromonad strains were mutagenized to auxotrophy with DES and evaluated. Reversion frequencies were too low to be detected under the test conditions. Quick transformation assays were performed in order to determine if all auxotrophic aeromonad strains could take up DNA from wild-type aeromonad strains under the test conditions. This quick test was sufficient in determining strong (31 or more colonies), intermediate (6-30 colonies), and weak (or no) (0-5 colonies) transformation capabilities (Figure 2.7). These designations were subjective and based on the appearance of the assay plates. Six of the auxotrophs were assayed in triplicate to demonstrate reproducibility. Furthermore, in nine of the strains, two or three different auxotrophic mutants were isolated from the same parent strain to evaluate potential variability of transformation results due to allele-specific effects. For example, three independently isolated mutants (a histidine auxotroph, a pyrimidine auxotroph, and a serine auxotroph) of strain 92 were tested. These results (Table 2.2) demonstrated that

there was no substantial variation between the different auxotrophic markers being transformed or between assays performed with the same isolate. The scope of transformability experiments indicated that not all aeromonad strains were capable of transformation; 20/46 (43%) could not be transformed under these test conditions (Table 2.2). None of the three *Aeromonas* ATCC strains were able to be transformed. The remaining 26/46 (57%) strains were considered to be competent for transformation. DNA from all of the aeromonads could transform at least one other competent strain. The clustering analysis indicated that the strains clustered into three major groups. This clustering solution yielded a cophenetic correlation coefficient of 0.8551. The closer the cophenetic correlation is to 1.000, the more closely the dendrogram of the cluster analysis preserves the original data. The three groups obtained from the cluster analysis roughly corresponded to their species designations based on *gyrB* sequences (Figure 2.8). The *A. veronii* isolates were considered to be the most transformable, with many of the isolates taking up DNA from 10 to 20 different strains (Table 2.2).

Discussion

These data demonstrate that some members of the *Aeromonas* genus are capable of natural transformation, but competence is induced under specific conditions. Not all aeromonads form transformant colonies, at least under the conditions tested in this study. *In vitro* competence of transformable strains occurs when they are cultivated in dilute growth medium. This observation was also noted by Sakai (28) with *A. salmonicida* transformation and suggests that, at some level, competence may be controlled by nutrient limitation. Induction of competence in *Aeromonas* occurs during late stationary

phase (Figure 2.1). This is in contrast to other transformable genera which become competent at different points during exponential phase and maintain competence for a shorter period than was found for *Aeromonas* (2, 6, 24, 36). The latter may represent a mechanism of competence induction and maintenance not previously described, possibly the involvement of specialized sigma factors. Moreover, this competence mechanism may reflect the aquatic environmental controls on population growth for *Aeromonas*.

Aeromonas transformation can only occur in the presence of sodium and either magnesium or calcium. Other bacteria, such as *Mycobacterium* (23), *Azotobacter vinelandii* (24), and *Bacillus subtilis* (26), require magnesium or calcium. *Bacillus subtilis* also requires sodium for transformation, but potassium or ammonium can replace sodium (26). A marine strain needed sodium, but it is unclear if this was a specific requirement for transformation or a general requirement for growth (11). In the natural habitats of the *Aeromonas* isolated for this study, the concentrations of sodium, magnesium, and calcium can reach the concentrations required in order for transformation to occur (Chapter 4), indicating that transformation may be possible outside of the lab. The optimal temperature for *Aeromonas* transformation is around 30ºC, but it can occur at lower and higher temperatures. Temperatures in the Lubbock playas have been measured in surface waters in the early morning from 2°C to 26°C depending on season (J. R. Huddleston, unpublished data) and presumably warming to higher temperatures during the day. No special buffers or defined pH are needed for the process to occur; high transformation frequencies can be obtained in distilled water that has been supplemented with sodium and magnesium (Figure 2.3), indicating that if

enough free DNA were present in aquatic habitats, transformation of aeromonads could probably occur.

Within the genus *Aeromonas* there are differing levels of transformability of strains and species, which has evolutionary implications. Each isolate in this study was identified to species using *gyrB* sequences. These identifications give some insight as to the nature of gene flow within and among the aeromonad groups. Some strains appear to act only as DNA donors, whereas others can act as both donors and recipients. Since 43% of strains in this study were not transformed, gene flow between aeromonads is not entirely unrestricted. However, all of the strains can act as donors. The structure of the transformation groups suggests that most competent aeromonads only take up DNA from closely related aeromonads. Nevertheless, more limited gene flow can also occur between transformation groups because there are a few strains within the aeromonad population that are able to take up DNA from more than one group (Table 2.2).

A similar study with soil isolates of *Pseudomonas stutzeri* (32) also found that transformability of different strains within a single species can vary. About two-thirds of the *P. stutzeri* strains were transformable, and levels of transformability of these strains differed from one another. Closely related strains had the same level of transformability. Differential intraspecific transformation has been reported in 4% of *Acinetobacter calcoaceticus* strains (8), 30% of *Helicobacter pylori* strains (12), and 42% of *Haemophilus influenzae* strains (27). These studies, including this one with *Aeromonas*, demonstrate that not all strains of a particular species are capable of transformation, and those that are capable often have differing transformation frequencies from one another.

Aeromonas strains 108 and 124 were capable of taking up DNA from donors in all of the transformation groups (Table 2.2). The reason for this is unknown but is reflective of the differing levels of transformation within a single species. It could be that these strains recognize uptake signal sequences from all of the aeromonad species or that there is a general uptake signal sequence that is found in all aeromonads that only these broadly transformable strains are capable of recognizing. Another possibility is that these strains have restriction–modification systems that function at a lower level, allowing more DNA from foreign sources to be sustained. This would normally not be beneficial for the bacterial cell unless it was in a situation where it would die anyway unless it took up DNA and adapted to the environment, even if the DNA was from very different sources. About 10% of the strains are not as broadly transformable as these two but can still take up DNA strongly from at least one other group. This cross-group gene flow has the potential to greatly affect the genetic structure of the entire aeromonad population. These strains can take up DNA from different groups and donate DNA to different groups, serving as an intermediate to disseminate genes from one group to another.

The lack of observed transformation of some of the isolates both within and between phenotypic complexes could be due to a variety of different factors. Impairment of any one of the multiple steps of transformation might lead to inefficient or complete lack of transformation. According to Lorenz and Wackernagel (20), impairments might include (1) lack of genetic capability by the recipient strain to bind and take up DNA; (2) inappropriate physiochemical conditions for competence induction or transformation; (3) lack of a specific nucleotide recognition sequence in the donor DNA; (4) presence of an

efficient restriction–modification system in the recipient, resulting in donor DNA destruction; (5) extensive mismatch correction following recombination, resulting in erasure of donor genetic information; and (6) inefficient homologous recombination due to local nucleotide sequence divergence between donor and recipient DNA. Whatever the cause of the lack of transformation, the results are the same: sexual isolation of some strains.

These noncompetent strains are isolated in that they do not receive new genetic material through transformation, yet they are able to disseminate their DNA among the population. In the event that deleterious alleles were to be distributed through the competent population by transformation, some of the broadly transformable strains could take up the DNA from these sexually isolated strains thereby restoring the allele in the transformation group and also to the population as a whole.

Transformation in aeromonads has both clinical and research implications. Antibiotic resistance could be transferred from one strain to another under favorable conditions. *In vitro* studies show that antibiotic resistance genes and not just amino acid biosynthetic genes can be transferred through natural transformation (25, 35). Virulence genes, relevant to human and fish pathogenesis, can also be transferred from one aeromonad to another, as Sakai (28) demonstrated with extracellular proteases in *A. salmonicida*. Transformation could also serve as a genetic system to manipulate the genome of aeromonads in research or for genetic mapping.

Extensive natural transformation could explain why the phylogeny and taxonomy of *Aeromonas* species are sometimes difficult to determine. Morandi et al. (22) demonstrated problems in constructing phylogenetic histories in *Aeromonas* and provided evidence for possible horizontal gene transfer.

The recently completed genome sequence of *A. hydrophila* ATCC 7966^T (not used in this study) indicates the presence of a competence/damage-inducible protein CinA and a DNA internalization-related competence protein ComEC/Rec2 (31). This strain also has genes that are necessary for transformation in other Gram-negative organisms, such as components of a type II secretion system (38) and type IV pili (7, 34). Preliminary research in our laboratory indicated that type IV pili are also essential for transformation in *Aeromonas* sp. strain 92 (Chapter 3), and further work is being done to determine what other gene products are involved.

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Table 2.1. Effect of different culture media on competence induction in strain C-70 as measured by transformation frequency at 24 h post-inoculation (mean ± SE). NB, nutrient broth; LB, Luria broth; TSB, tryptic soy broth; GMB + his, NCE glucose minimal broth supplemented with 1 m*M* histidine. Transformation frequencies were determined by dividing the number of transformants by the initial number of cells added per assay (4.0×10^7 cells). A transformation frequency of $(8.33 \pm 11.8) \times 10^{-9}$ transformants $(\text{recipient cell})^{-1}$ indicates that one transformant appeared on one of the three replicate assay plates. This is the lowest transformation frequency that can be measured using this protocol.

Media	Transformants $(\text{recipient cell})^{-1}$
20% NB	$(3.38 \pm 0.20) \times 10^{-4}$
40% NB	$(1.32 \pm 0.12) \times 10^{-5}$
60% NB	$(3.33 \pm 3.12) \times 10^{-8}$
80% NB	0
100% NB	0
20% LB	$(8.33 \pm 11.8) \times 10^{-9}$
100% LB	0
20% TSB	$(8.33 \pm 11.8) \times 10^{-9}$
100% TSB	$(8.33 \pm 11.8) \times 10^{-9}$
20% GMB + his	0
100% GMB + his	0

Table 2.2. Scope of transformability of environmental aeromonads using quick transformation assays. Crude DNA from each wild-type (prototrophic) donor strain was added to each auxotrophic recipient strain to test for uptake of DNA. Numbers in the boxes refer to the number of transformant colonies that appeared for each assay. A "+" indicates there were too many colonies to count, presumably over 50. The assay results were color-coded for ease of visualizing the data only: 0-5 colonies, weak or no transformation (white squares); 6-30 colonies, intermediate transformation (gray squares); 31 or more colonies, strong transformation (black squares). These are arbitrary designations. In the auxotrophy column, a slash (/) between represents different isolates with single auxotrophies, and "and" represents a single isolate with double auxotrophies. Species identifications given are based on the best match to *gyrB* sequences deposited in the NCBI database. Groupings are based on cluster analysis.

Table 2.2. Continued.

Auxotrophic DNA recipients / Prototrophic DNA Donors

Unique number	Species identification	Auxotrophy	68	74	85	440	70	79	92	96	124	86	97	90	91	108	ATCC 9071	69	93	67	121	122	88	71	82	63	158	98	78	64	87	119	80	102	89	107	112	118	117	123	ATCC 7965	113	441	115	106	ATCC 1546	111	120
68	*A. salmonicida*	arginine	0	0	0	1	0	0	0	0	0	0	0	0	0	0	4	0	5	0	0	3	0	0	0	0	0	0	0	0	12	12	0	1	0	1	0	0	8	7	0	5	0	0	0	0	9	2
74	*A. salmonicida*	tryptophan	2	1	10	4	3	3	7	4	6	10	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
85	*A. salmonicida*	histidine	50	50	50	50	50	50	50	50	50	50	50	12	1	1	0	2	0	2	3	1	0	2	0	3	3	0	0	1	0	21	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
440	*A. veronii* bv. Sobria	aromatics/tryptophan	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
70	*A. veronii*	methionine	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
79	*A. salmonicida* subsp. *pectinolytica*	uracil	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
92	*A. salmonicida* subsp. *pectinolytica*	histidine/pyrimidine/se	50	50	50	14	50	50	50	50	50	50	50	38	2	0	0	0	0	2	1	4	2	1	0	2	4	0	1	6	0	5	0	0	1	0	0	0	2	2	1	1	2	1	0	3	4	0
96	*A. salmonicida* subsp. *salmonicida*	threonine	0	0	0	4	1	1	0	0	2	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
124	*A. veronii*	histidine/aromatics	50	50	50	50	50	50	50	50	50	50	50	50	11	12	7	5	8	3	21	20	18	13	7	5	5	12	10	7	0	50	6	14	16	12	7	13	7	20	15	14	13	10	10	5	17	7
86	*A. salmonicida*	phenylalanine, tyrosine	0	0	9	0	50	50	50	50	50	50	50	0	2	0	2	1	0	17	25	18	50	6	0	0	3	2	0	0	8	26	1	8	0	0	0	0	0	0	4	10	5	3	0	0	3	30
97	*A. salmonicida*	proline	0	1	3	0	0	0	1	1	5	0	14	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
90	*A. bestiarum*	threonine/uracil	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
91	*A. hydrophila* subsp. *hydrophila*	threonine	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
108	*A. veronii*	pyrimidine	17	23	27	28	19	29	15	14	12	19	16	22	50	50	50	43	39	47	50	45	50	27	4	39	30	27	37	16	0	36	38	44	50	25	24	39	47	21	33	15	18	32	43	15	18	7
ATCC 15468(T)	*A. caviae*	tryptophan	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
93	*A. encheleia*	threonine	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
69	*A. hydrophila* subsp. *hydrophila*	threonine	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
67	*A. hydrophila*	histidine/threonine	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
121	*A. hydrophila* subsp. *hydrophila*	threonine	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
122	*A. veronii*	threonine	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
88	*A. hydrophila*	methionine	0	1	0	0	0	0	0	0	0	0	1	0	0	0	2	2	1	1	0	0	2	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
71	*A. media*	aromatics	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
82	*A. hydrophila*	histidine	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
63	*A. media*	histidine	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	2	0	1	7	9	7	7	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
158	*A. veronii* bv. Sobria	tryptophan	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
98	*A. media*	glutamate	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
78	*A. salmonicida*	histidine	0	0	3	0	2	0	0	0	0	0	0	0	0	2	0	0	0	0	0	2	0	11	0	50	50	50	50	50	2	0	0	1	0	1	0	1	2	0	0	0	1	0	0	1	0	0
64	*A. media*	aromatics	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	25	25	21	50	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
87	*A. veronii* bv. Sobria	pyrimidine	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
119	*A. veronii*	histidine	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
80	*A. veronii*	tryptophan	0	0	0	0	1	0	4	0	0	0	0	0	0	0	0	0	0	2	1	0	0	0	0	0	0	0	1	0	31	0	50	50	32	20	17	29	29	36	0	50	50	50	50	50	40	50
102	*A. veronii*	serine	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	34	32	0	12	12	11	12	18	9	10	16	8	7	13	11	5
89	*A. veronii*	uracil	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	8	0	50	50	50	50	50	50	50	50	50	50	50	50	50	44	50	50
107	*A. veronii* bv. Sobria	threonine	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	13	13	12	13	17	10	14	8	13	13	10	9	18	5	5	18
112	*A. veronii* bv. Sobria	threonine	0	0	0	0	0	0	0	1	1	0	1	2	2	0	0	1	1	1	0	0	3	0	0	1	0	0	0	0	0	1	0	0	6	1	0	3	0	1	0	1	2	2	0	0	1	0
118	*A. veronii*	serine	1	1	1	4	0	0	0	0	2	8	0	2	2	1	3	1	0	1	1	1	1	0	2	0	1	1	0	0	3	1	50	50	37	50	50	50	50	50	50	50	50	50	50	50	50	14
117	*A. veronii* bv. Sobria	methionine/serine/trypt	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	1	0	5	21	0	15	11	11	24	7	3	7	11	19	14	35	16	0
123	*A. veronii* bv. Sobria	histidine	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ATCC 7965	*A. hydrophila*	methionine/histidine	2	2	5	0	6	6	1	2	3	5	0	1	1	1	0	3	0	3	1	4	1	1	1	0	0	0	0	1	0	1	20	15	16	20	28	23	18	23	25	20	14	13	41	17	5	35
113	*A. veronii* bv. Sobria	histidine	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	2	0	0	0	1	0	2	3	0	0	0	0	0
441	*A. veronii* bv. Sobria	histidine/tryptophan	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	4	0	0	1	0	0
115	*A. veronii* bv. Sobria	aromatics	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	2	1	4	0	0	0	2
106	*A. veronii* bv. Sobria	pyridoxine/histidine	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ATCC 9071	*A. veronii* bv. Sobria	histidine	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	5	11	5	8	4	6	4	5	4	2	6	10	1	4	8	3
111	*A. veronii* bv. Sobria	tryptophan	1	0	1	0	0	1	0	0	0	0	1	0	1	0	0	0	0	1	1	0	3	0	2	0	0	0	0	0	0	1	1	7	3	4	3	2	1	2	2	5	4	2	2	5	34	15
120	*A. veronii*	histidine	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	6	0	1	0	3	1	2	4	1	2	2	4	5	2	3

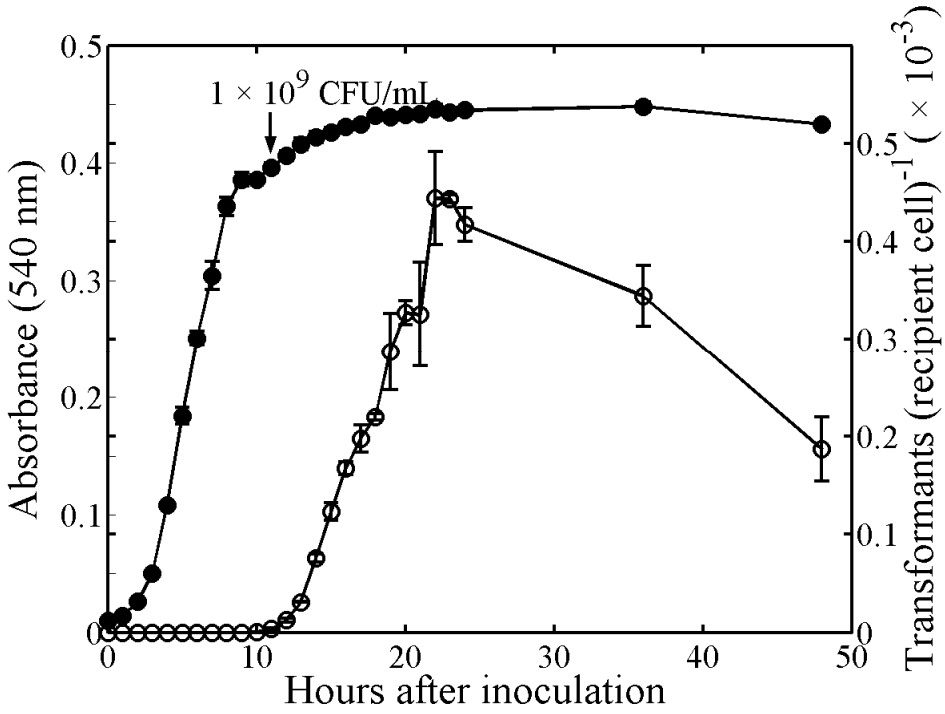

Figure 2.1. Effect of growth phase on competence induction. Symbols: (●) Absorbance at 540 nm, (○) Transformation frequency, (↓) Beginning of stationary phase (equivalent to 1×10^9 CFU ml^{-1}), (n = 3), error bars indicate ± 1 SE.

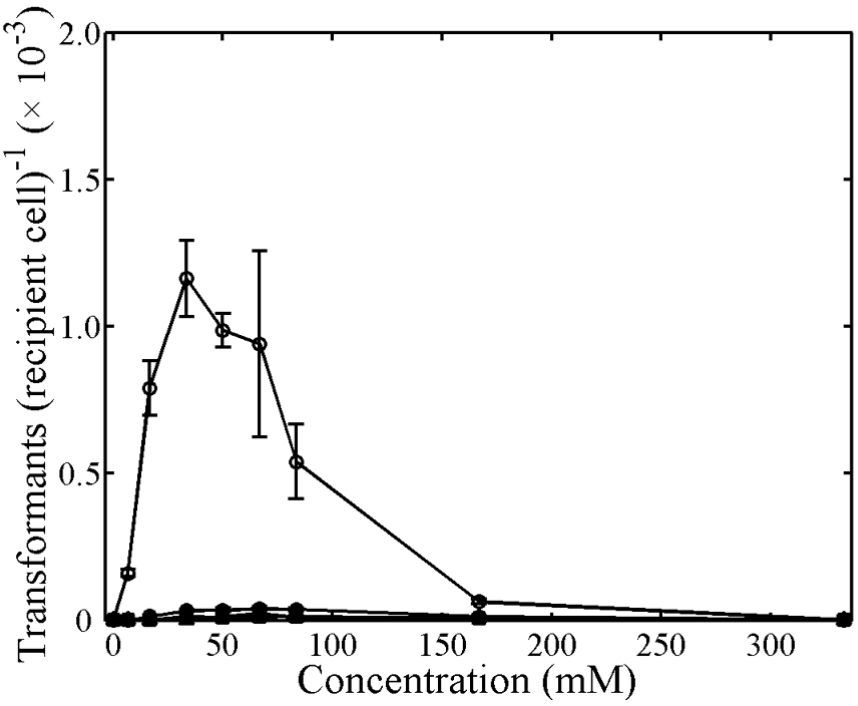

Figure 2.2. Effect of monovalent cation concentrations on transformation frequency. Symbols: (○), varying NaCl + 13 m*M* $MgSO_4$, (●) varying KCl + 13 m*M* $MgSO_4$, (■) varying LiCl + 13 m*M* $MgSO_4$, (Δ) varying NH_4Cl + 13 m*M* $MgSO_4$, Values are means (n = 4) ± 1 SE.

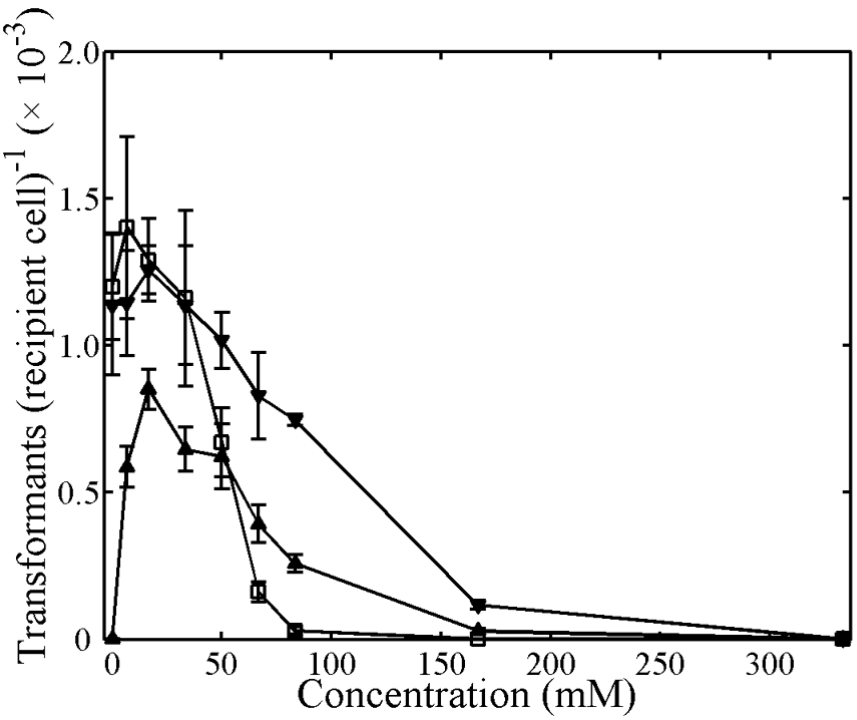

Figure 2.3. Effect of divalent cation and buffer concentrations on transformation frequency. Symbols: (□) Varying $CaCl_2$ + 33 m*M* NaCl, (▲), varying $MgSO_4$ + 33 m*M* NaCl, (▼) varying Tris + 33 m*M* NaCl + 13 m*M* $MgSO_4$. Values are means (n = 4) ± 1 SE.

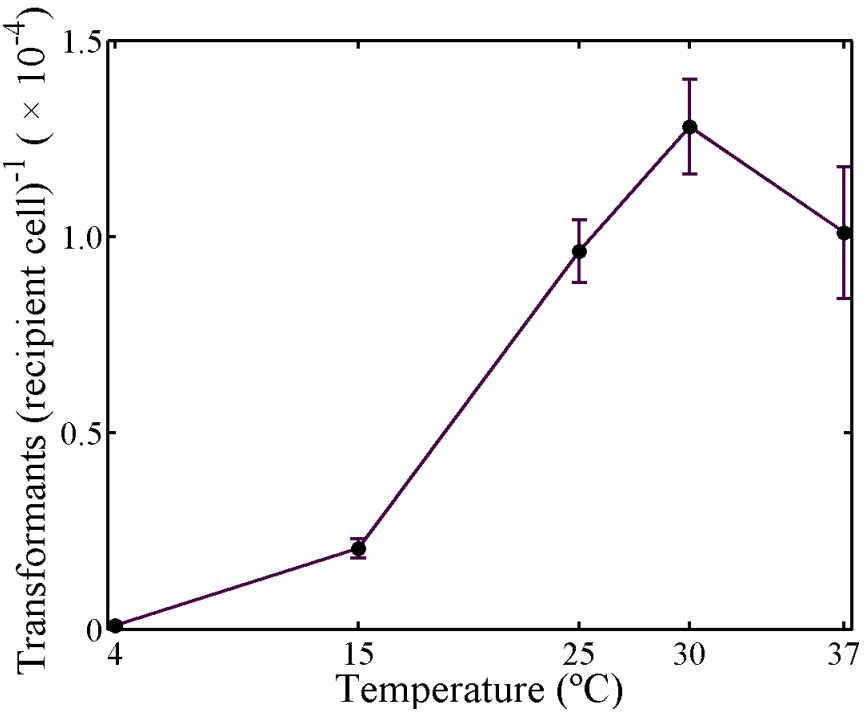

Figure 2.4. Effect of temperature on transformation frequency. Values are means (n = 4) ± 1 SE.

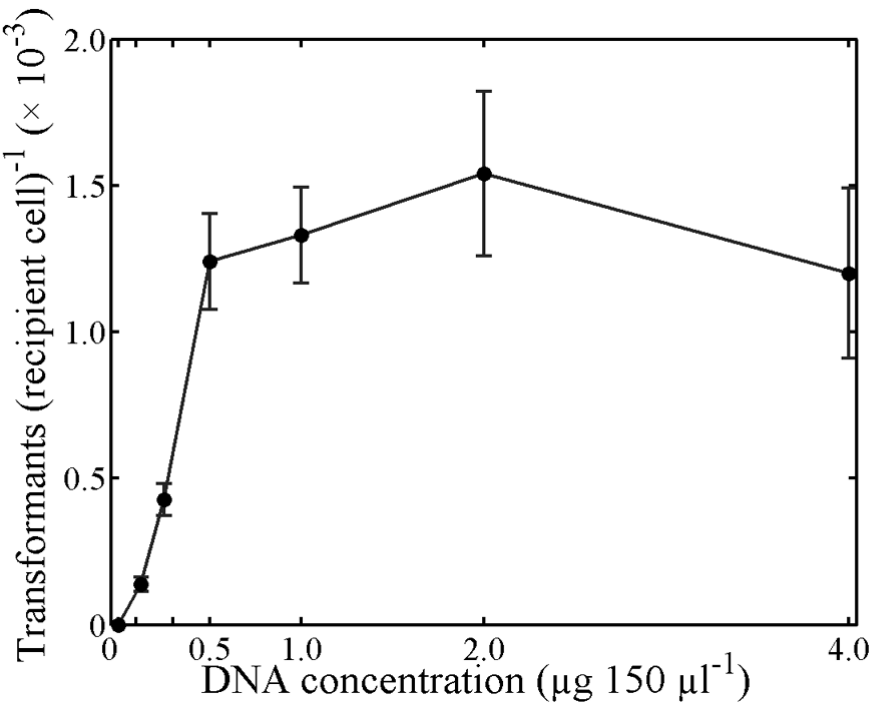

Figure 2.5. Effect of DNA concentration on transformation frequency. Values are means (n = 4) ± 1 SE.

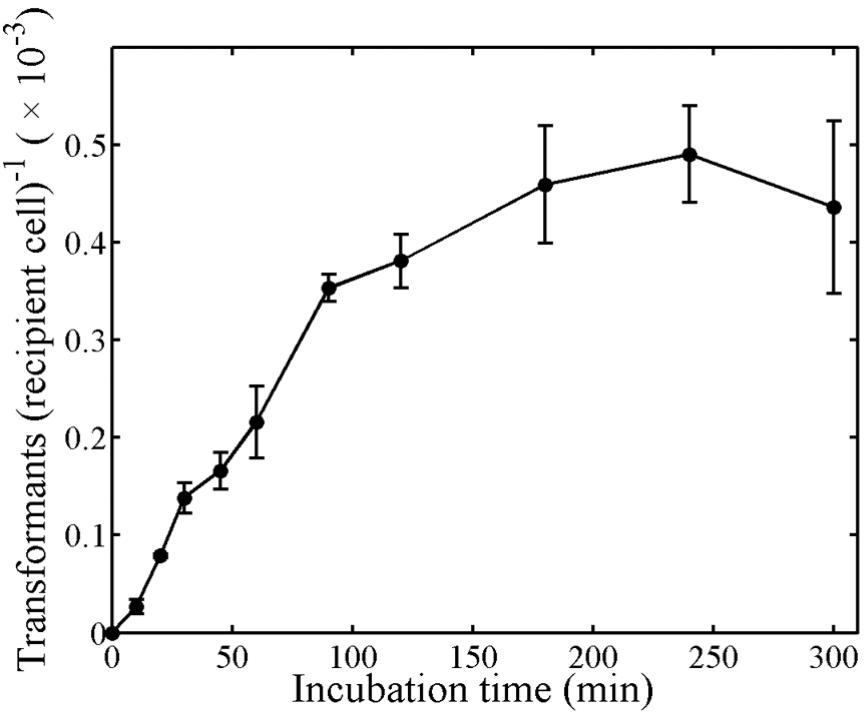

Figure 2.6. Effect of DNA incubation time prior to DNase I addition. Competent cells were incubated with DNA for the indicated times, DNase I was added, and the assays were incubated for an additional 60 min before plating. Values are means (n = 4) ± 1 SE.

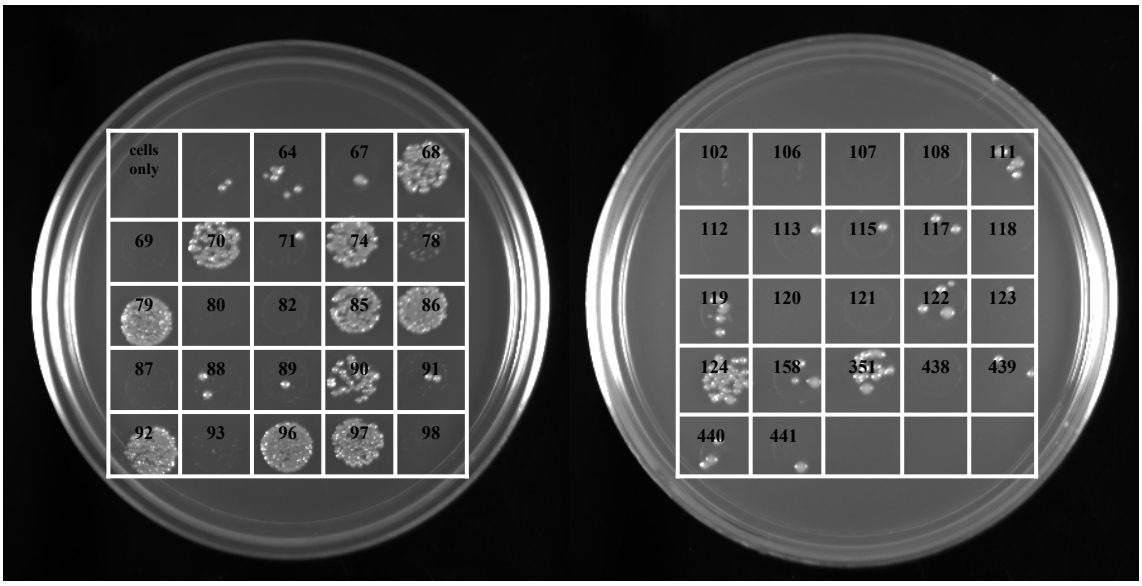

Figure 2.7. *Aeromonas* C-70 quick transformation assays on NCE glucose minimal agar. Each square represents one assay in which crude DNA from a different wild-type (prototrophic) donor strain was added to assay buffer with competent C-70 (auxotrophic) recipient cells. Each tested donor strain is designated by its strain number. Cells only, *Aeromonas* C-70 assay with no added DNA, is shown in the upper left corner of Plate 1.

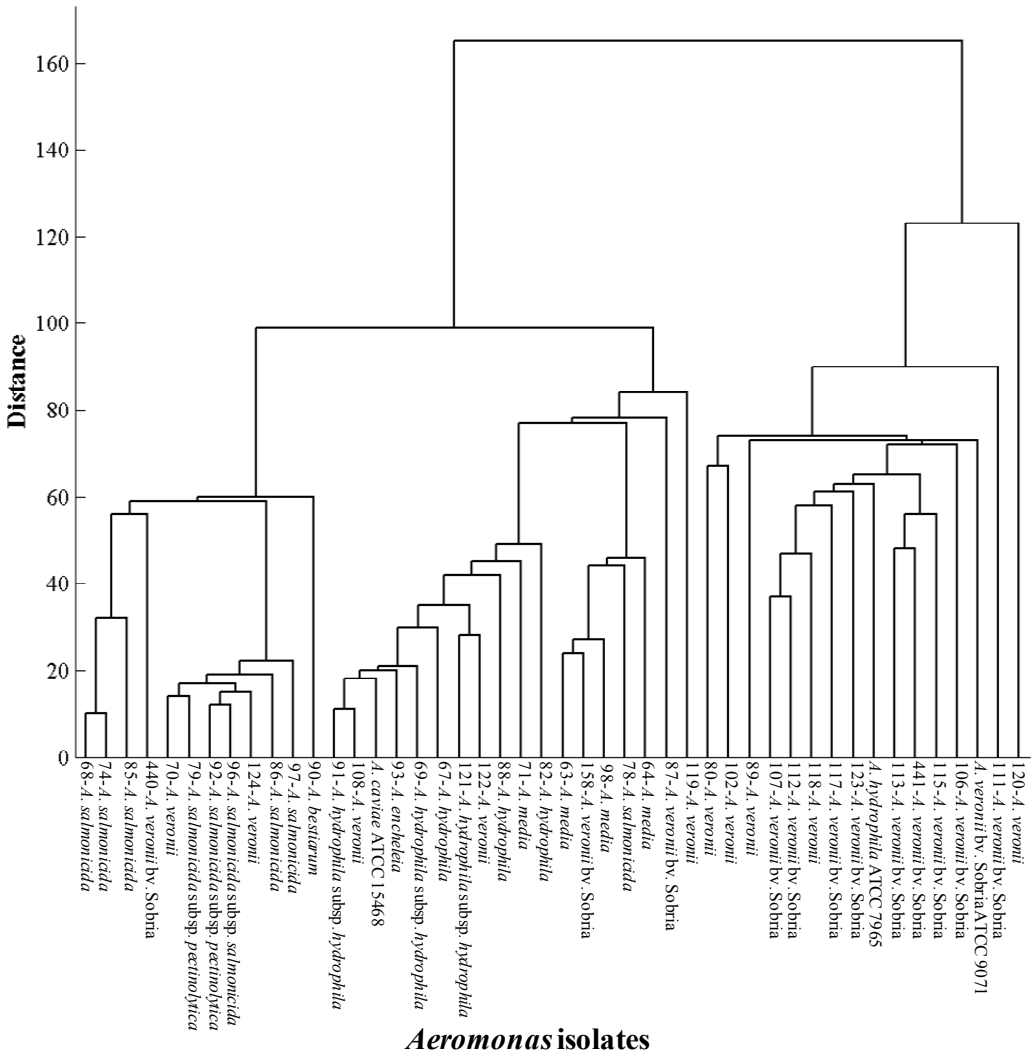

Figure 2.8. Transformation groups dendrogram results of cluster analysis based on the numbers of transformants obtained in the scope of transformability assays. Analysis was done using Matlab (version 6.0.0.88 Release 12) and assuming independent observations, underlying normal distributions, and homoscedasticity.

CHAPTER III

GENES INVOLVED IN AEROMONAD TRANSFORMATION

Abstract

Natural genetic transformation in *Aeromonas* species has recently been characterized, but the genes involved in this process are unknown. This study identifies four genes necessary for transformation in *Aeromonas—tapY1* which encodes a type IV pili biogenesis protein, *ptsI* which encodes the phosphoenolpyruvate phosphotransferase protein I, *recBC* which encodes a helicase/exonuclease involved in recombination, and *clpA/clpS* which encodes a caseinolytic protease. These genes indicate mechanisms of competence development through starvation (*ptsI*) and stress responses (*clpA/clpS*), DNA binding (*tapYI*), and recombination (*recBC*).

Introduction

Aeromonads are ubiquitous aquatic bacteria capable of causing opportunistic and intestinal infections in humans. In Chapter 2, the optimal physiological parameters of natural transformation in these organisms were described. No genes important for transformation in *Aeromonas* have been identified up to this point. Identifying several of the genes involved in transformation is necessary in order to fully understand competence and transformation, and hence gene flow, in *Aeromonas* species.

There are many essential genes in the process of transformation. The process consists of competence induction, DNA binding, fragmentation, uptake, integration, and

finally expression of the transformed DNA sequences. Some of the genes involved in each of these steps are shown in Table 1.1 of Chapter 1. The goal of this study was to identify genes important in aeromonad transformation.

Materials and Methods

Mutagenesis of *Aeromonas* isolate. A putative *Aeromonas salmonicida* isolate that was previously mutagenized to histidine auxotrophy (C-70, a derivative of strain 92) and characterized for transformation in Chapter 2 was used in all experiments. DNA from the wild-type (prototrophic) strain was used as donor DNA. Plasposon mutagenesis according to the protocol of Dennis and Zylstra (9) was performed using tri-parental matings in order to obtain random insertional mutants. The plasposon (pTn*Mod*-OGm) was propagated in donor strain *Escherichia coli* GM2163 (*dcm6 dam13*::Tn*9*) (New England Biolabs, Beverly, Ma.) 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 (12) were grown in Luria broth overnight. Five-hundred microliters of each strain were combined and microcentrifuged (Eppendorf centrifuge 5418) at 14,000 rpm (16,873 × *g*) for 5 minutes at room temperature. The pelleted cells were suspended in 50 of µl NCE buffer (4) and placed in a small pool on a Luria agar plate. The mating plate was incubated for 8 h at 37°C, and the cells were washed off the plate with 3 ml of NCE buffer, diluted, plated onto Luria agar with 30 µg/ml of gentamycin (Sigma-Aldrich, St. Louis, Mo.) and 30 µg/ml of ampicillin (Sigma), and incubated overnight at 30°C to recover mutagenized transconjugant *Aeromonas* cells.

Screening for transformation-negative mutants. In order to induce competence, gentamycin-resistant transconjugants were transferred to 20% Difco nutrient agar (NA) (Becton Dickinson, Sparks, Md.) adjusted to pH 7.5 with 1 *N* NaOH and incubated at 30°C for 48 h. Crude DNA was extracted from the prototrophic donor as described by Juni (15), and 400 µl were spread onto a plate of no citrate E (NCE) minimal medium (4) supplemented with 0.25% glucose (Sigma). 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 transformation using quantitative transformation assays as previously described in Chapter 2 and were also tested for adequate growth in glucose NCE supplemented with histidine. If isolates demonstrated reduced growth in glucose NCE with histidine, they were assayed using DNA from a parent strain that is spontaneously resistant to nalidixic acid to determine if the isolates were also transformation deficient.

Sequencing of DNA from transformation-negative mutants. Genomic DNA was extracted from transformation-negative mutants using the method of Ulrich and Hughes (35), digested with SalI (Promega, Madison, Wi.), ligated with T4 DNA ligase (Promega), and electroporated into *E. coli* JM109 using standard protocols. Plasmid DNA was extracted using the Wizard Plus SV Minipreps DNA Purification System (Promega). The plasmids were sequenced at the Core Facility of the Texas Tech University Center for Biotechnology and Genomics using primers based on the sequences of Schmidt-Eisenlohr et al. (30) but modified for the gentamycin plasposon: Ori5seq (5′-GCC TTT TGC TCA CAT GTT CTT TCC-3′) and Ori3seq(modified) (5′-ATT CGA

GCT CTT AAT TAA TTT AAA TC-3′). Sequences were analyzed using Vector NTI, 9.0.0 (Invitrogen, Carlsbad, Ca.) and were compared against all microbe sequences deposited in NCBI using BLAST (1).

PCR amplification. Copies of the mutated genes were amplified from the mutant strains with the designated primers (Table 3.1). The following cycling conditions were used with a Robocycler (Stratagene, La Jolla, Ca.): 1 cycle of 95˚C for 1 min, 30 cycles of 95˚C for 30 sec, 50˚C for 30 sec, 72˚C for 1 min 15 sec, and 1 cycle of 72˚C for 5 min. Amplification reactions contained 1 × PfuUltra II reaction buffer (Stratagene), 2.5 m*M* of each dNTP, 1.0 µl of PfuUltra II Fusion HS DNA polymerase (Stratagene), 0.1 µ*M* of each primer (Integrated DNA Technologies, Inc., Coralville, Ia.), and 0.1 µg of template DNA in a final reaction volume of 50 µl. The resulting fragments were purified by combining 45 µl of PCR product, 32.5 µl of 40% polyethylene glycol 8000 (Amresco, Solon, Oh.), 10 µl of 3 *M* sodium acetate, pH 5.2, and 12.5 µl of sterile distilled water and incubating for 15 min at room temperature followed by microcentrifugation (16,873 × *g*, 14,000 rpm) for 15 min at room temperature. The DNA pellet was washed with 70% ethanol, dried, and dissolved in 20 µl of water. It was then diluted to 20 ng/µl in sterile distilled water.

Transforming PCR products with mutated sequences into wild-type *Aeromonas*. Transformation assays were performed as previously described in Chapter 2 with a few modifications. Competent *Aeromonas* cells (histidine auxotroph, sensitive to nalidixic acid) were prepared by inoculating a single colony into 20% nutrient broth (NB) (Becton Dickinson), pH 7.5, and incubating with aeration overnight at 30ºC. The culture

was diluted 1:100 in 20% NB, and incubated again with aeration for 24 ± 2 h at 30ºC. Transformations were carried out in buffer consisting of 36.7 m*M* Tris HCl (Sigma) and 16.8 m*M* Trizma base (Sigma), pH 7.86, 25 m*M* $MgSO_4 \cdot 7H_2O$ (Spectrum, Gardena, Ca.), and 50 m*M* NaCl (EM Science, Gibbstown, N.J.). Competent cells were added in a volume of 40 µl (4.0×10^7 cells) along with 10 µl of diluted PCR product. The transformation mixture was incubated at 30ºC for 1 h. After the initial incubation, transformation was stopped by adding 10 µl of 200 µg/ml deoxyribonuclease (DNase) I (Sigma) solution and then incubating at 30ºC for 1 h to degrade free DNA. Controls were performed by adding DNase I to the assay mixture before the initial 1-h incubation to eliminate all transformation. No-DNA, no-cell, and no-DNA/no-cell assays were also performed as negative controls. Transformation mixtures were inoculated onto Luria agar with 30 µg/ml of gentamycin and incubated 48 h at 30ºC. Three transformant colonies for each of the PCR products were then subcultured and assayed for loss of transformation. Quantitative transformation assays were performed as above except that DNA with a stock concentration of 100 ng/µl from a prototrophic, nalidixic acid-resistant strain was used. Assays were set up with five replicates each and plated onto Luria agar with 50 mg/l of nalidixic acid (Sigma) and onto NCE glucose minimal agar.

Assays for loss of phenotypes. The strains transformed with the mutant PCR products were tested for loss of function of the target genes. The *tapY1* mutants were evaluated for loss of the type IV pili through visualizing the cells with transmission electron microscopy. Approximately 8-10 colonies of each strain were re-suspended in 1 ml each of sterile, distilled water. Bacterial cells were adsorbed onto Formvar (Ted Pella,

Redding, Ca.)-coated copper grids (ranging in size from 75 to 250-mesh) (Gilder, Grantham, Lincolnshire, England). Excess liquid was wicked off of the grids before they were negatively stained with 1% uranyl acetate (Fisher, Pittsburg, Pa.) for 30 min. Grids were then washed in 70% EtOH and sterile distilled water before being examined with a Hitachi H-8100 transmission electron microscope (Tokyo, Japan) at 75 kV.

The *ptsI* mutants and wild-type were inoculated into 2 ml of NCE glucose broth supplemented with histidine. The tubes were incubated overnight at 30°C with aeration, and then the absorbance (λ = 600 nm) was read. Lack of growth or reduced growth indicated that glucose utilization had been affected due to loss of PtsI protein function.

The *recBC* mutants and wild-type strain were grown in 2 ml of tryptic soy broth (Becton Dickinson) at 30°C overnight, inoculated onto tryptic soy agar (TSA) (Becton Dickinson) with five replicates, and exposed to ultraviolet light from a General Electric (Cleveland, Oh.) ultraviolet tube (G815) 30 cm above the plate for 0 sec, 10 sec, 20 sec, and 30 sec. The plates were incubated at 30°C overnight and observed for absence of growth on the plates, indicating a loss of Rec protein function (40).

The *clpA*/*clpS* mutants were grown in 20% NB, pH 7.5, overnight and diluted in 0.85% NaCl, and five replicates of TSA with 80 ml/L of sterile skim milk were inoculated. The plates were incubated at 30°C overnight and observed for pinpoint colonies, indicating a loss of Clp protein function.

Results

Four mutants deficient in transformation were obtained using the plasposon mutagenesis procedure. Sequences of the mutations revealed the genes *ptsI*, *tapY1*, *recBC*, and *clpA/clpS* are important for transformation in this aeromonad. Table 3.2 shows the similarity of gene sequences of the mutants to sequences deposited in NCBI. The defective genes from these mutants were amplified by PCR and transformed into the histidine auxotroph sensitive to nalidixic acid. Gentamycin-resistant transformants were subsequently tested for transformation to histidine prototrophy and nalidixic acid resistance, and failure to obtain such colonies was interpreted as loss of the ability to transform DNA. Both types of transformations were done in order to demonstrate that the loss of transformation was not due to a secondary phenotypic effect, i.e., glucose-negative mutants will always appear as though they have lost the ability to transform in the histidine transformation assays on glucose minimal medium, and so the nalidixic acid transformation assays will indicate whether or not transformation ability was really lost. The data showed that all of the PCR transformants either had lost or were greatly reduced in their ability to be transformed when compared to the wild type (Table 3.3 and Table 3.4).

Transmission electron micrographs were taken of the wild-type strain and the *tapY1* plasposon mutant and PCR mutants (Figure 3.1). Only the wild-type displayed type IV pili. The transformants mutagenized with an interrupted *ptsI* gene demonstrated reduced utilization of glucose compared to the wild-type control strain, although the decrease in growth was less pronounced in the PCR transformants when compared to the

initial plasposon mutant (Figure 3.2). The transformants mutagenized with interrupted *recBC* genes were found to be more sensitive to ultraviolet light than the wild-type control strain at 10, 20, and 30 sec of exposure (Figure 3.3). The transformants mutagenized with interrupted *clpA/clpS* genes formed pinpoint colonies on TSA with casein as opposed to the wild-type strain forming colonies 2 mm in diameter after 24 h (Figure 3.4).

Discussion

Three of the four genes [*ptsI*, *tapY1*(homologue of *pilY1*), and *recBC*] implicated as important for transformation in *Aeromonas* in this study have been associated with transformation in other competent bacterial genera (Table 1.1 in Chapter 1). This is the first time *clpA/clpS* involvement in transformation has been reported. The donor DNA is taken up from the environment, processed, and is integrated into the recipient chromosome through the mechanism of homologous recombination. RecBCD is essential for transformation due to its role in recombination. It is a helicase/exonuclease complex (exonuclease V in *E. coli*) that degrades linear duplex DNA (21) and is essential in recombination of linear DNA molecules in *E. coli* (20). The complex is composed of three protein subunits—RecB (134 kDa), RecC (129 kDa), and RecD (67 kDa) (21). The complex has two ATP-dependent exonuclease activities. One is a dsDNA exonuclease that degrades linear duplex DNA and the other is ssDNA exonuclease that degrades linear ssDNA. The complex also acts as a DNA helicase (21). As RecBCD is unwinding and degrading duplex DNA from one end of the DNA, it recognizes Chi sites (5'-GCTGGTGG-3') and then switches to degrading only the 5' strand. It loads RecA onto

the 3' strand, forming the nucleoprotein filament so that homologous recombination is able to proceed. RecBCD has been shown to play a role in the transformation of *Acinetobacter baylyi* (18), *Haemophilus influenzae* (19), and *Neisseria gonorrheae* (24). Mutants without this complex, as in the aeromonad in this study, have no or greatly reduced transformability.

The protein encoded by *tapYI* (type IV *Aeromonas* pilus) is a type IV pilin biogenesis protein. Other competent Gram-negative genera also require type IV pili in order for transformation to occur. It has been proposed that the role of type IV pili in transformation is to bind donor DNA to the recipient cell as occurs in *Pseudomonas aeruginosa* (36). *Actinobacillus actinomycetemcomitans* (38), *Dichelobacter nodosus* (17), *Eikenella corrodens* (33), *Haemophilus influenzae* (37), *Moraxella catarrhalis* (22), *Neisseria gonorrhoeae* (29, 31), *Neisseria meningitidis* (13), *Pseudomonas stutzeri* (14), and *Synechoscystis* sp. (41) demonstrate decreased competence in the absence of functional type IV pili biogenesis proteins. It is unknown as to whether type IV pili themselves affect transformation or whether the proteins involved in type IV pili assembly also play a separate role in transformation. In *Neisseria meningitidis*, the PilQ protein forms the channel that PilE subunits pass through in order to form the type IV pilus fibers. DNA binds strongly to PilQ, but only weakly to the type IV pili (2). Other competent bacteria, such as *B. subtilis*, do not have type IV pili, but they must have prepilin-like proteins [ComGC, ComGD, ComGE, and ComGG (8)] in order for transformation to occur. *Streptococcus pneumoniae* and *H. influenzae* also must have proteins similar to those involved in type IV pili systems yet do not express type IV pili

(3). Conversely, competent *Acinetobacter baylyi* (formerly BD413) express pili, but they are not required for transformation (28). From this study, it is evident that this strain of *Aeromonas* also requires type IV pili biogenesis proteins in order to be transformable. As with other competent bacteria, more research will have to be done in order to elucidate the molecular relationship between *Aeromonas tapY1* and transformation.

The protein encoded by *ptsI* is Enzyme I (EI) of the phosphoenolpyruvate: carbohydrate phosphotransferase system. Enzyme I transfers a phosphate group from phosphoenolpyruvate to the histidine protein (HPr). These two proteins are active in the phosphotransferase systems of substrates including, but not limited to, glucose. Enzyme II (EIIA) then transfers the phosphate from HPr to glucose, forming glucose-6-phosphate. Under low-glucose conditions, there is an accumulation of phosphorylated EIIA which activates adenylate cyclase, causing levels of cAMP to rise which in turn activates catabolite activator protein (Crp) (10). Crp has many regulatory functions, including catabolite repression and competence induction in *Haemophilus influenzae* (5). PtsI mutants of *H. influenzae* (23) and *Neisseria meningitidis* (32) demonstrate a reduction or elimination of transformation. This could be due to lack of competence induction because EI cannot activate Crp. In this *Aeromonas* strain, EI is required for transformation to occur.

The ATPases encoded by the *clp* (caseinolytic protease) genes fall within the AAA+ superfamily of ATPases associated with various cellular activities. The Clp proteins act as molecular chaperones that prevent aggregation, refold and remodel, and degrade proteins (27) damaged under stress conditions (39). In *E. coli*, ClpA functions as

the ATP-binding protein in a complex with ClpP protease, forming ClpAP which degrades polypeptides (16). ClpS is an accessory protein that targets substrates for degradation by ClpAP (11, 42). ClpA is able to function as a molecular chaperone in the absence of ClpP (27). Other closely related Clp proteins are ClpB, ClpC, ClpE, ClpL, ClpV, ClpX, and ClpY (HsIU) (43).

ClpP and ClpC have been implicated in competence development in both *B. subtilis* (25, 26) and *S. pneumoniae* (6, 7). In *S. pneumoniae* ClpP negatively regulates the transcription of the *comCDE* operon under conditions that do not favor competence (7). However, in *B. subtilis* ClpP activates ComK which allows for the expression of the late *com* genes (25). MecA binds to ComK, and ClpC binds to MecA. ComK activity is inhibited when bound to these proteins. Under appropriate conditions ComS is expressed, which releases ComK from the complex. ComK activates its own transcription and that of the *comCDE* operon. During the escape from competence, ComK is degraded by ClpP along with ClpC (34). This is the first report of the involvement of ClpA/ClpS in transformation for any competent bacterial strain.

In Chapter 2, it was observed that *Aeromonas* becomes competent under nutrient-limiting conditions and in the stationary phase of growth. This evidence, along with the genes identified in this study, lays the foundation for a model of competence development in *Aeromonas*. As nutrients are depleted, the phosphotransferase system is needed in order to induce carbohydrate catabolite repression and increase cAMP. Cyclic AMP could then induce competence genes that are unidentified at this time. It is unknown if ClpA and ClpS work with ClpP to degrade proteins or if they are functioning

alone as a molecular chaperone in the development of competence and transformation in *Aeromonas*. If the former is true, then ClpAP could be active under conditions of stress, such as starvation, allowing for induction of competence genes. More research should be done to elucidate the relationship between ClpA/ClpS and transformation in *Aeromonas*.

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Table 3.1. Primers used for PCR amplification of various mutated genes in transformation mutants.

Gene	Primer	Sequence	Wild-type product length
tapY1	Forward	5′ CGC GAA TTC ATG CTGA CAT CAT GGA AAA CTG TGG TTG ATG 3′	3612 bp
tapY1	Reverse	5′ CGC TCT AGA TCA TTG GGG CTC ATC CTC ATA CAG G 3′	
clpA/clpS	Forward	5′ CGC AAG CTT TTA GTG GGA GAC AGC TTC AAT ATT GAC CTG 3′	2630 bp
clpA/clpS	Reverse	5′ CGC GGA TCC ATG AGC AAG CAG AAA GAA CTC TTT GC 3′	
ptsI	Forward	5′ CGC GAA TTC ATG ATA TCT GGC ATT CTT GCA TCC CCC 3′	1728 bp
ptsI	Reverse	5′ CGC TCT AGA TTA GCA GAC TGC CTT CTG CTT AAT AAA G 3′	
recBC	Forward	5′ CGC GAA TTC TCA TGC CTC TAC CTC GTT CAA TGG G 3′	3621 bp
recBC	Reverse	5′-CGC TCT AGA ATG GCT AAC CCG CTC AAT ACC CTG CG-3′	

Table 3.2. Transformation-defective mutants.

Gene	GenBank Accession (range)	Functional homologue	% Identity
tapY1	CP000644 (704882-708493)	Type IV pilin biogenesis protein, *A. salmonicida*	98
ptsI	CP000644 (3295323-3297050)	Phosphoenolpyruvate-protein phosphotransferase, *A. salmonicida*	94
recBC	NC_009348 (4317599-4321219)	Exodeoxyribonuclease V, gamma subunit and beta genes, *A. salmonicida*	95
clpA/clpS	NC_009348 (2609146-2611775)	ATP-dependent Clp protease, ATP-binding subunit ClpA, ATP-dependent Clp protease adaptor protein ClpS, *A. salmonicida*	96

Table 3.3. Transformation frequencies of the histidine marker into the wild-type strain, plasposon mutants, and strains transformed with mutant PCR products. The lowest detectable transformation frequency by the assay is 2.50×10^{-8} transformants (recipient cell^{-1}). (n = 5, ±1 SE)

Strain	Transformation frequency [transformants (recipient cell^{-1})]
Wild-type	$(2.17 \pm 0.14) \times 10^{-3}$
tapY1 plasposon mutant	$< 2.50 \times 10^{-8}$
tapY1 PCR mutant 1	$< 2.50 \times 10^{-8}$
tapY1 PCR mutant 2	$< 2.50 \times 10^{-8}$
tapY1 PCR mutant 3	$< 2.50 \times 10^{-8}$
ptsI plasposon mutant	$< 2.50 \times 10^{-8}$
ptsI PCR mutant 1	$< 2.50 \times 10^{-8}$
ptsI PCR mutant 2	$< 2.50 \times 10^{-8}$
ptsI PCR mutant 3	$< 2.50 \times 10^{-8}$
recBC plasposon mutant	$< 2.50 \times 10^{-8}$
recBC PCR mutant 1	$< 2.50 \times 10^{-8}$
recBC PCR mutant 2	$< 2.50 \times 10^{-8}$
recBC PCR mutant 3	$< 2.50 \times 10^{-8}$
clpA/clpS plasposon mutant	$(4.88 \pm 0.54) \times 10^{-6}$
clpA/clpS PCR mutant 1	$< 2.50 \times 10^{-8}$
clpA/clpS PCR mutant 2	$< 2.50 \times 10^{-8}$
clpA/clpS PCR mutant 3	$< 2.50 \times 10^{-8}$

Table 3.4. Transformation frequencies of the nalidixic acid resistance marker into the wild-type strain, plasposon mutants, and strains transformed with mutant PCR products. The lowest detectable transformation frequency is 2.50×10^{-8} transformants (recipient cell^{-1}). (n = 5, ±1 SE)

Strain	Transformation frequency [transformants (recipient cell^{-1})]
Wild-type	$(8.85 \pm 0.96) \times 10^{-4}$
tapY1 plasposon mutant	$< 2.50 \times 10^{-8}$
tapY1 PCR mutant 1	$< 2.50 \times 10^{-8}$
tapY1 PCR mutant 2	$(2.00 \pm 0.65) \times 10^{-7}$
tapY1 PCR mutant 3	$< 2.50 \times 10^{-8}$
ptsI plasposon mutant	$(5.04 \pm 0.30) \times 10^{-6}$
ptsI PCR mutant 1	$< 2.50 \times 10^{-8}$
ptsI PCR mutant 2	$< 2.50 \times 10^{-8}$
ptsI PCR mutant 3	$< 2.50 \times 10^{-8}$
recBC plasposon mutant	$< 2.50 \times 10^{-8}$
recBC PCR mutant 1	$< 2.50 \times 10^{-8}$
recBC PCR mutant 2	$< 2.50 \times 10^{-8}$
recBC PCR mutant 3	$< 2.50 \times 10^{-8}$
clpA/clpS plasposon mutant	$(1.30 \pm 0.60) \times 10^{-7}$
clpA/clpS PCR mutant 1	$< 2.50 \times 10^{-8}$
clpA/clpS PCR mutant 2	$< 2.50 \times 10^{-8}$
clpA/clpS PCR mutant 3	$< 2.50 \times 10^{-8}$

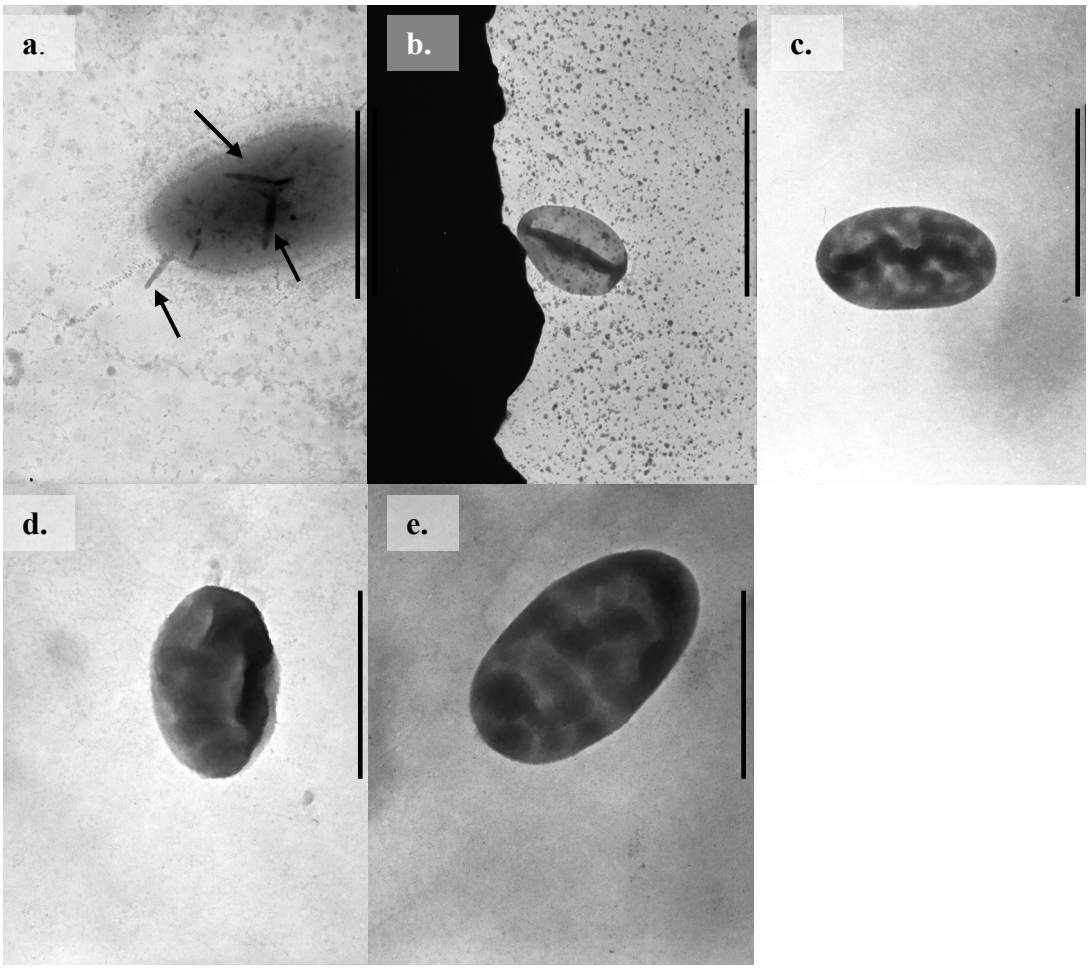

Figure 3.1. Transmission electron micrographs showing presence and absence of type IV pili. a.) Wild-type with pili (arrows), bar = 303 nm. b.) *tapY1* plasposon mutant with no pili, bar = 454 nm. c.) *tapY1* PCR mutant 1 with no pili, bar = 454 nm. d.) *tapY1* PCR mutant 2 with no pili, bar = 303 nm. e.) *tapY1* PCR mutant 3 with no pili, bar = 303 nm.

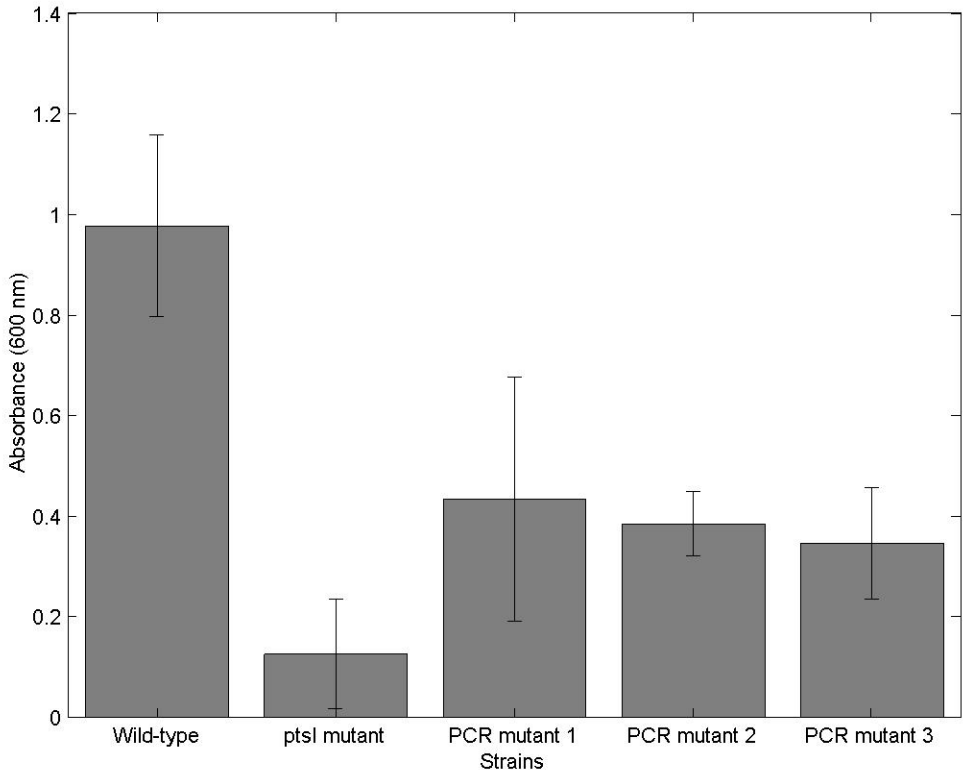

Figure 3.2. Glucose utilization of strains. Growth under conditions that require glucose utilization by the wild-type *ptsI*$^{+}$ strain, plasposon *ptsI* mutant, and the three PCR transformant *ptsI* mutants as measured by absorbance at λ = 600 nm. (n = 5, ±1 SE)

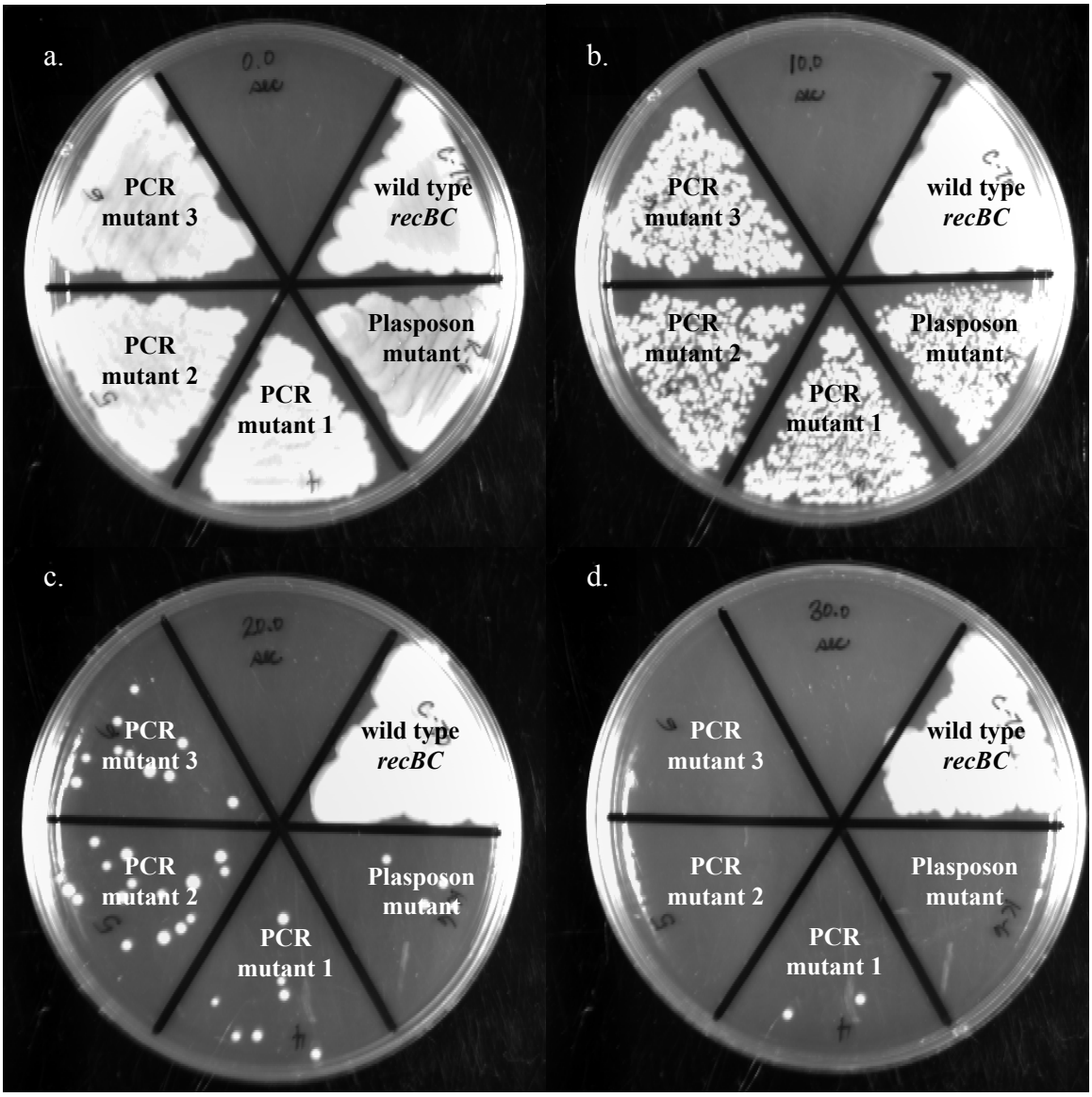

Figure 3.3. Growth of *recBC* mutants and the wild-type strain after ultraviolet light exposure. a) 0 sec of exposure. b) 10 sec of exposure. c) 20 sec of exposure. d) 30 sec of exposure. The plates shown are representative of the results of five replicates.

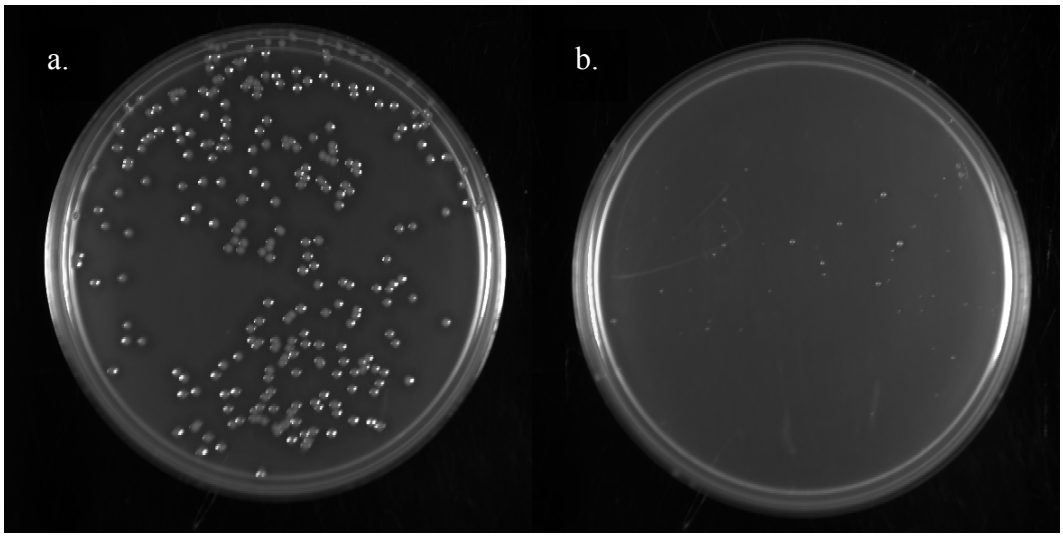

Figure 3.4. Growth of a *clpA/clpS* mutant and the wild-type strain after 24 h on TSA with casein. The plates shown are representative of the results of five replicates. a) Wild-type colonies 2 mm in diameter. b) Pinpoint colonies of *clpA/clpS* mutant. (All plates of the *clpA/clpS* mutants appeared the same, but are not shown here.)

CHAPTER IV

AEROMONAD TRANSFORMATION IN PLAYA WATER

Abstract

Aeromonas transformation assays were done in playa water in order to better understand the potential for transformation under natural conditions. The water from three Lubbock, Texas playa lakes—Maxey, Higinbotham, and Stevens—was collected monthly for one year and filter-sterilized prior to assay. The water samples were analyzed for their levels of bicarbonate, boron, calcium, carbonate, chloride, conductivity, magnesium, nitrate nitrogen, pH, phosphorus, potassium, sodium, sulfate, sodium absorption ratio, and total dissolved solids and compared to the number of transformants obtained in order to determine which factors affect transformation significantly in the environment. Multiple regression analyses indicated that 56.2% of the transformation variation was due to the measured chemical components with the strongest correlation to bicarbonate (Pearson's correlation coefficient = -0.311, p = 0.033). Transformation frequencies were lower in the water when compared to optimal transformation buffer, yet still took place, indicating the process could occur in the lakes and serve as a natural environment for gene exchange.

Introduction

Natural transformation in *Aeromonas* has been described under laboratory conditions. It is of interest to determine if aeromonads are able to undergo this process in

one of their natural habitats—the water column in playa lakes. It has been shown that *Aeromonas salmonicida* can be transformed in sediment (18), but not in the water column. *Aeromonas* species have been easily isolated from playa lakes in the Lubbock, Texas region (9, 22, 23). Playa lakes are small circular to oval basins collect surface runoff waters from the surrounding area. There are more than 20,000 playa basins on the High Plains of Texas, New Mexico, Colorado and Oklahoma (6). Several studies have been conducted to analyze both the chemical and biological composition of urban playa lakes (1, 8, 13). The composition of the playas is influenced by the quality of surface-water runoff, which is directly related to the way the land in the watershed is being used, whether it be an urban lake or rural lake (7).

Studies have been done with other competent bacterial species to determine if transformation could occur in their natural habitats. *Bacillus subtilis* could be transformed with chromosomal DNA at a low level [4.0×10^{-7} transformants (recipient $cell^{-1}$)] in a groundwater microcosm (17) and also in a soil microcosm [up to 1.1×10^{-1} transformants (recipient $cell^{-1}$)] (5). Unlike *Bacillus*, *Vibrio* was capable of yielding higher transformation frequencies with plasmid DNA in non-sterile seawater [1.8×10^{-7} transformants (recipient $cell^{-1}$)] as opposed to non-sterile marine sediments [0 transformants (recipient $cell^{-1}$)] from Joulter's Cay, Bahamas (15). Studies with pseudomonads in soil microcosms demonstrated that *Pseudomonas stutzeri* was transformed with chromosomal DNA [2.6×10^{-7} transformants (recipient $cell^{-1}$)] (20), and *Pseudomonas fluorescens* was transformed with plasmid DNA [5.8×10^{-8} transformants (recipient $cell^{-1}$)] (3). *Enterococcus faecalis* was able to be transformed

with plasmid DNA [10^{-8} transformants (recipient $cell^{-1}$)] in microcosms in activated sludge basins and anoxic sludge digestors in a sewage water treatment plant (12). *Acinetobacter calcoaceticus* was transformed with chromosomal DNA in biofilms set up in a river [7.76×10^{-6} transformants (recipient $cell^{-1}$)] (24).

Several of these studies found that transformation of the recipient strain was made possible or enhanced by the addition of nutrients (14, 15) or ions (17). The goal of this study was not to attempt to add components to enhance transformation but to determine if transformation of *Aeromonas* could occur in its natural habitat and which, if any, of the chemical components naturally present in the playa lake water, if any, might contribute to the transformation of *Aeromonas* species under natural conditions.

Materials and Methods

Sampling and filtration of water. Samples from three urban playa lakes (Higinbotham, Maxey, and Stevens) in Lubbock, Texas were taken monthly from October 2006 through September 2007. Surface-water samples were taken from the banks of the playa. A 500-ml plastic cup attached to a 2-meter pole was disinfected with 0.5% Amphyl (National Laboratories, Montvale, N.J.), wiped, and then dipped into the lake water three times to rinse it. The samples were taken and transferred to a 1-L sterile, clean, Pyrex bottle. The temperature of each sample was taken with a laboratory thermometer and recorded. The samples were kept on ice until they could be processed in the lab. Portions of the samples were sent chilled with ice packs to Waters Agricultural Laboratories, Inc. (Owensboro, Ky.) for analysis of levels of bicarbonate,

boron, calcium, carbonate, chloride, conductivity, magnesium, nitrate nitrogen, pH, phosphorus, potassium, sodium, sulfate, sodium absorption ratio, and total dissolved solids. The remaining water was centrifuged at 48,200 × *g* (Sorvall RC-5C Plus centrifuge, 20,000 rpm in an SS-34 rotor) for 30 min in order to remove particulates. The supernatant was then filtered through a 0.45-µm cellulose nitrate membrane filter (Micro Filtration Systems, Dublin, Ca.) and allowed to warm to room temperature before being used in transformation assays.

Quantitative transformation assays. Quantitative transformation assays were performed as previously described in Chapter 2 with a few modifications. Competent *Aeromonas* cells (histidine auxotroph) were prepared by inoculating a single colony into 20% nutrient broth (NB) (Becton Dickinson, Sparks, Md.), pH 7.5, and incubating with aeration overnight at 30ºC. The culture was diluted 1:100 in 20% NB and incubated again with aeration 24 ± 2 h at 30ºC. Quantitative transformation assays were performed in filtered playa water or 100 µl of transformation buffer consisting of 36.7 m*M* Tris HCl (Sigma-Aldrich, St. Louis, Mo.) and 16.8 m*M* Trizma base (Sigma), pH 7.86, 25 m*M* $MgSO_4 \cdot 7H_2O$ (Spectrum, Gardena, Ca.), and 50 m*M* NaCl (EM Science, Gibbstown, N.J.). Competent cells were added in a volume of 40 µl (4.0×10^7 cells) to each assay. Pure DNA from the wild-type aeromonad (prototroph) was prepared according to the method of Ulrich and Hughes (21). One µg of DNA was added in a volume of 10 µl (final concentration in assay of 6.7 ng/µl). The assay mixture was incubated at 30ºC for 1 h. After the initial incubation, transformation was stopped by adding 10 µl of 200 µg/ml deoxyribonuclease (DNase) I (Sigma) solution and then incubating at 30ºC for 1 h to

degrade free DNA. Controls were performed by adding DNase I to the assay mixture before the initial 1 h incubation to eliminate all transformation. No-DNA, no-cell, and no-DNA/no-cell assays were also performed as negative controls. All assays were set up with five replicates. Assays were diluted in 0.85% NaCl, spread onto no citrate E (NCE) glucose minimal agar (2), and incubated 48 h at 30ºC. Colonies that grew on NCE glucose minimal agar were considered to be transformants. Transformants were counted and data analyzed with multiple regression analyses, ANOVAs, and Pearson's LSD post-hoc tests using SPSS for Windows, Release 9.0.0 (SPSS Inc., Chicago, Il.). The numbers of transformants per assay were used in all analyses.

Results

Assays versus controls. Pearson's LSD post-hoc tests [using the Bonferroni correction (10)] showed that the means of transformants in the incomplete assays used as controls (water only, water with DNA, water with cells) were significantly different from those of the complete assays (water with DNA and cells), but not significantly different from one another (Table 4.1). These results also demonstrated that there was no difference in transformation between the incomplete assays of water with cells and the complete assays of water with DNA and cells, indicating there was not sufficient free DNA in the playa water to allow for significant transformation of the added *Aeromonas* cells.

Differences in transformation, chemical components, lakes, and date of sampling. ANOVAs were used to compare the lakes ignoring the date of sampling, to

compare the measured chemical components from the water ignoring the date of sampling, and to compare transformation and the chemical components together ignoring the date of sampling. The results showed there was a significant difference between the transformation measured in each lake, the chemical components measured in each lake, and transformation in each lake with respect to the chemical components measured in each lake (Table 4.2). Another ANOVA was done in order to determine if there was a difference in transformation over time (Table 4.3). The Greenhouse-Geisser correction was used since Mauchly's test showed sphericity was violated (4). The results of this analysis showed there was a significant difference between lakes over time, date of sampling with regard to lake, date of sampling with regard to chemical components, and date of sampling with regard to lake, chemical components, and date of sampling. Multiple comparisons using Pearson's LSD post-hoc tests with the Bonferroni correction (10) showed that there were significant differences between the means of the numbers of transformants per assay obtained from the different lakes (Table 4.4).

Effects of chemical components on transformation. Transformation assay data and data over chemical components of the water were collected for each of the lakes at each sampling date (Table 4.5). The year-long trends for each of the chemical components measured are shown in Figure 4.1 through Figure 4.15. The water collected from Stevens lake in November showed peaks of chloride, boron, magnesium, sodium, sulfate, potassium, total dissolved solids, conductivity, and calcium where the other lakes did not demonstrate this trend. Transformation peaked in January for all of the lakes, with Higinbotham water yielding the highest numbers of transformants [780

transformants per assay, equivalent to 1.95×10^{-5} transformants (recipient cell^{-1})] obtained for any of the lakes or sampling dates. Throughout the year, Higinbotham water generally yielded the most transformants, followed by Maxey water, and then Stevens water (Figure 4.16). Transformation always occurred at a lower level in the water samples than in the optimal transformation buffer [$(1.31 \pm 0.7) \times 10^{4}$ transformants per assay, equivalent to $(3.28 \pm 1.75) \times 10^{-4}$ transformants (recipient cell^{-1})].

In order to determine which, if any, of the measured chemical components in the water might affect transformation, a multiple regression analysis using the forced entry method was performed (Table 4.6). Conductivity was eliminated from the model because of its collinearity with other variables. The R^2 value was 0.562, indicating the measured chemical components in the water account for 56.2% of the variability seen in the number of transformants obtained in the assays. The Pearson correlation matrix and its significance values (Table 4.7) were examined to determine which of the measured chemical components of the water were responsible for the variation in transformation. The only significant correlation between transformation and any of the chemical components was with bicarbonate (Pearson's correlation coefficient = -0.311, $p = 0.033$). No other significant interactions were identified with the other measured chemical components and transformation.

Discussion

The regression analysis indicated that the majority of the variation in transformation could be attributed to the measured chemical components and that

bicarbonate can inversely influence transformation. The Pearson correlation coefficient, although statistically significant, was low at -0.311, indicating a weak relationship. More research will have to be done with a greater number of water samples and controlled laboratory experiments in order to determine the exact effect of bicarbonate on transformation.

The regression model does not account for 43.8% of the transformation variation. Playa lakes are dynamic systems where every environmental variable cannot be measured. The transformation variation is likely due to variables that were not investigated in this study, such as effects from rainfall, from the presence of migratory waterfowl, particularly the thousands of Canada geese that winter in the Lubbock area, and from runoff. The waste products of Canada geese can change the water composition considerably (11). They can also add new microbes to the water system, such as *Salmonella* sp., that could influence the concentration of microbial mass and also free DNA. The runoff that drains into these urban playa lakes contains a complex mixture of chemicals including pesticides, hydrocarbons, and fertilizers, just to name a few (13, 25). All of these factors could influence competence development of the aeromonads as well as DNA binding and subsequent transformation steps.

Although transformation in the water samples was low compared to the optimal buffer controls, transformation occurred at a measurable frequency. This suggests that water in the natural habitat of aeromonads contains sufficient ions, such as magnesium, calcium, and sodium, and has an appropriate pH for transformation to occur. (See

Chapter 2 for optimal transformation conditions.) Natural transformation in lake water, especially low-nutrient oligotrophic lake water (16), could serve as a physical location for gene transfer to take place among aquatic bacteria, specifically *Aeromonas* species. It is possible that *Aeromonas* species become competent and even remain competent indefinitely in the natural habitat. (See Chapters 2 and 3 for discussions of competence induction in *Aeromonas*.)

This study did not detect any transformants in the assays with water and cells only. There was not sufficient DNA either in quality or quantity, present in the water to transform the added *Aeromonas* cells. In reality, the majority of aeromonad transformation may not be happening in the water column but rather in association with sediments in the aquatic environment, since DNA can be protected from degradation when it is adsorbed to sand, silt, or clay (17). This has been demonstrated *in vitro* with *Aeromonas salmonicida* (18, 19).

Another possibility is that much of aeromonad transformation could be occurring in biofilms. Williams et al. (24) demonstrated that *Acinetobacter calcoaceticus* cells are capable of transformation within biofilms formed in river epilithon. More research should be done in this area to determine if aeromonad transformation occurs at different frequencies in biofilms compared to that in free water.

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Table 4.1. Multiple comparisons of complete (water + cells + DNA) and incomplete assays (water + DNA, water + cells, water) using Pearson's LSD Post-hoc tests based on observed means.

$Assay_I$	$Assay_J$	Mean difference ($Assay_I$- $Assay_J$)	Standard error	Significance (p)	95% confidence interval	
					Lower bound	Upper bound
water + cells + DNA	water + DNA	54.84[a]	2.76	< 0.001	49.29	60.39
	water + cells	54.72[a]	2.76	< 0.001	49.18	60.27
	Water	54.79[a]	2.76	< 0.001	49.24	60.34
water + DNA	water + cells + DNA	-54.849[a]	2.76	< 0.001	-60.39	-49.29
	water + cells	-0.12	2.76	0.966	-5.67	5.43
	Water	-0.04	2.76	0.987	-5.59	5.51
water + cells	water + cells + DNA	-54.72[a]	2.76	< 0.001	-60.27	-49.17
	water + DNA	0.12	2.76	0.966	-5.43	5.67
	Water	0.072	2.76	0.979	-5.48	5.62
Water	water + cells + DNA	-54.79[a]	2.76	< 0.001	-60.34	-49.24
	water + DNA	-0.04	2.76	0.987	-5.51	5.59
	water + cells	-0.07	2.76	0.979	-5.62	5.48

[a] The mean difference is significant at the 0.05 level.

Table 4.2. ANOVA results comparing transformation in lakes ignoring date of sampling, measured chemical components in the water ignoring date of sampling, and transformation and chemical components together ignoring the date of sampling.

Source	TypeIII Sum of Squares	df	Mean square	F	Significance (*p*)
Intercept	138666.80	1	138668.80	202.21	< 0.001
Lake	54019.80	2	27009.90	39.39	< 0.001
Components	405192.50	3	135064.20	196.96	< 0.001
Lake, components	167198.40	6	27866.41	40.64	< 0.001
Error	32915.70	48	685.74		

Table 4.3. ANOVA results comparing transformation in lakes over time, date of sampling with regard to lake, date of sampling with regard to chemical components, and date of sampling with regard to lake, chemical components, and date of sampling. The Greenhouse-Geisser correction was used.

Source	TypeIII Sum of Squares	df	Mean square	F	Significance (*p*)
Date of sampling	374384.90	1.92	195108.70	82.60	< 0.001
Date of sampling, lake	285588.20	3.84	74416.38	31.50	< 0.001
Date of sampling, components	1115323.00	5.76	193748.30	82.02	< 0.001
Date of sampling, lake, components	863547.10	11.51	75005.49	31.75	< 0.001
Error	217565.50	92.11	2362.15		

Table 4.4. Multiple comparisons of transformation obtained from the three different lakes—Maxey, Higinbotham, and Stevens—using Pearson's LSD Post-hoc tests based on observed means.

$Lake_I$	$Lake_J$	Difference between means ($Lake_I$- $Lake_J$)	Standard error	Significance (p)	95% confidence interval	
					Lower bound	Upper bound
Maxey	Higinbotham	-13.63[a]	2.39	< 0.001	-18.44	-8.83
	Stevens	7.26[a]	2.39	0.004	2.46	12.07
Higinbotham	Maxey	13.63[a]	2.39	< 0.001	8.83	18.44
	Stevens	20.90[a]	2.39	< 0.001	16.09	25.70
Stevens	Maxey	-7.26[a]	2.39	0.004	-12.07	-2.46
	Higinbotham	-20.90[a]	2.39	< 0.001	-25.70	-16.09

[a] The mean difference is significant at the 0.05 level.

Table 4.5. Transformation and chemical component data collected from October 2006 to September 2007 for each lake.

Lake	Sampling Date	Mean ± 1 SE Transformants per 150 µl assay	Bicarbonate (ppm)	Boron (ppm)	Calcium (ppm)
Maxey	October 2006	4.80 ± 1.60	10.67	0.030	27.88
Higinbotham	October 2006	36.00 ± 11.59	10.07	0.020	25.13
Stevens	October 2006	12.40 ± 3.20	11.29	0.050	30.89
Maxey	November 2006	24.00 ± 32.35	13.11	0.050	35.52
Higinbotham	November 2006	25.60 ± 24.01	10.67	0.031	27.36
Stevens	November 2006	6.00 ± 6.69	23.48	0.210	66.98
Maxey	December 2006	101.20 ± 49.06	14.95	0.041	40.92
Higinbotham	December 2006	118.00 ± 20.82	12.81	0.039	34.07
Stevens	December 2006	22.00 ± 8.39	14.33	0.045	37.61
Maxey	January 2007	188.00 ± 15.44	28.97	0.023	33.96
Higinbotham	January 2007	760.00 ± 169.12	25.01	0.043	29.71
Stevens	January 2007	92.40 ± 5.63	28.67	0.030	37.62
Maxey	February 2007	40.80 ± 31.66	247.05	0.057	39.21
Higinbotham	February 2007	14.00 ± 11.93	213.50	0.036	27.81
Stevens	February 2007	1.20 ± 1.60	292.80	0.035	39.75
Maxey	March 2007	2.00 ± 1.26	186.06	0.043	24.64
Higinbotham	March 2007	0.00	167.75	0.048	20.91
Stevens	March 2007	4.40 ± 3.88	183.00	0.040	25.62
Maxey	April 2007	72.00 ± 13.86	178.43	0.027	21.08
Higinbotham	April 2007	58.40 ± 10.98	158.60	0.017	18.72
Stevens	April 2007	18.40 ± 4.89	189.10	0.014	20.08
Maxey	May 2007	60.00 ± 14.14	183.00	0.041	24.20
Higinbotham	May 2007	96.80 ± 86.37	152.50	0.036	18.79
Stevens	May 2007	23.20 ± 6.01	204.35	0.043	28.41
Maxey	June 2007	5.20 ± 4.83	149.45	0.039	24.24
Higinbotham	June 2007	40.00 ± 77.04	149.49	0.031	19.30
Stevens	June 2007	0.00	210.45	0.033	30.42
Maxey	July 2007	1.20 ± 2.40	170.80	0.043	21.52
Higinbotham	July 2007	7.60 ± 4.08	176.90	0.028	23.28
Stevens	July 2007	3.20 ± 1.60	170.80	0.043	21.52
Maxey	August 2007	4.80 ± 2.04	237.90	0.044	46.23
Higinbotham	August 2007	12.40 ± 5.85	164.70	0.022	25.37
Stevens	August 2007	8.80 ± 5.46	189.10	0.017	25.36
Maxey	September 2007	56.40 ± 10.38	114.68	0.047	31.23
Higinbotham	September 2007	48.40 ± 8.14	80.52	0.037	20.95
Stevens	September 2007	9.20 ± 8.54	103.09	0.025	29.61

Table 4.5. Continued.

Lake	Sampling Date	Carbonate (ppm)	Chloride (ppm)	Conductivity (mmhos/cm)	Magnesium (ppm)
Maxey	October 2006	0	9.0	0.183	4.12
Higinbotham	October 2006	0	9.0	0.166	2.18
Stevens	October 2006	0	18.0	0.200	2.96
Maxey	November 2006	0	18.0	0.213	5.00
Higinbotham	November 2006	0	13.0	0.176	2.98
Stevens	November 2006	0	257.0	1.195	25.24
Maxey	December 2006	0	19.0	0.236	5.32
Higinbotham	December 2006	0	17.0	0.230	3.97
Stevens	December 2006	0	17.0	0.228	4.14
Maxey	January 2007	0	41.0	0.276	4.11
Higinbotham	January 2007	0	22.0	0.223	4.85
Stevens	January 2007	0	26.0	0.269	3.77
Maxey	February 2007	12	97.0	0.423	6.99
Higinbotham	February 2007	0	26.0	0.211	3.61
Stevens	February 2007	0	48.0	0.337	4.68
Maxey	March 2007	0	21.0	0.205	3.05
Higinbotham	March 2007	0	41.0	0.124	2.16
Stevens	March 2007	0	36.0	0.209	2.75
Maxey	April 2007	0	15.5	0.151	2.09
Higinbotham	April 2007	0	3.0	0.108	1.21
Stevens	April 2007	0	15.5	0.146	1.53
Maxey	May 2007	0	4.0	0.139	2.42
Higinbotham	May 2007	0	2.0	0.099	1.49
Stevens	May 2007	0	13.0	0.205	2.62
Maxey	June 2007	15	9.0	0.141	2.07
Higinbotham	June 2007	0	4.0	0.109	1.26
Stevens	June 2007	0	9.0	0.170	2.18
Maxey	July 2007	12	9.0	0.146	2.42
Higinbotham	July 2007	0	10.0	0.128	1.39
Stevens	July 2007	12	9.0	0.146	2.42
Maxey	August 2007	3	21.0	0.258	4.77
Higinbotham	August 2007	12	4.0	0.141	1.45
Stevens	August 2007	0	9.0	0.150	1.81
Maxey	September 2007	0	22.0	0.243	4.41
Higinbotham	September 2007	0	5.0	0.139	2.02
Stevens	September 2007	0	10.0	0.171	2.16

Table 4.5. Continued.

Lake	Sampling Date	Nitrate (ppm)	pH	Phosphorous (ppm)	Potassium (ppm)
Maxey	October 2006	3.50	7.8	0.110	3.49
Higinbotham	October 2006	4.00	7.5	0.070	4.43
Stevens	October 2006	3.50	7.7	0.110	5.06
Maxey	November 2006	1.50	7.9	0.009	3.43
Higinbotham	November 2006	5.00	7.7	0.008	4.86
Stevens	November 2006	1.00	7.3	0.085	12.23
Maxey	December 2006	1.50	7.7	0.068	4.21
Higinbotham	December 2006	5.00	7.6	0.110	6.52
Stevens	December 2006	3.00	7.5	0.190	7.31
Maxey	January 2007	0.30	7.9	0.031	4.01
Higinbotham	January 2007	0.15	7.7	0.130	7.66
Stevens	January 2007	0.22	7.8	0.150	7.74
Maxey	February 2007	0.10	8.4	0.480	9.31
Higinbotham	February 2007	0.14	7.7	0.046	6.16
Stevens	February 2007	0.53	7.9	0.250	10.31
Maxey	March 2007	0.10	8.0	0.091	3.62
Higinbotham	March 2007	0.10	7.4	0.270	4.13
Stevens	March 2007	1.13	8.0	0.140	5.01
Maxey	April 2007	1.59	8.1	0.057	3.34
Higinbotham	April 2007	0.74	7.3	0.110	4.15
Stevens	April 2007	1.29	8.1	0.062	4.05
Maxey	May 2007	0.95	8.1	0.120	3.05
Higinbotham	May 2007	0.56	7.5	0.380	3.74
Stevens	May 2007	0.88	7.6	0.080	3.95
Maxey	June 2007	0.20	8.7	0.040	1.09
Higinbotham	June 2007	0.10	7.6	0.090	1.24
Stevens	June 2007	0.30	7.7	0.051	2.08
Maxey	July 2007	0.30	8.4	0.058	2.35
Higinbotham	July 2007	0.30	7.3	0.250	2.22
Stevens	July 2007	0.30	8.4	0.058	2.35
Maxey	August 2007	2.63	7.9	0.670	5.81
Higinbotham	August 2007	0.12	8.6	0.210	3.74
Stevens	August 2007	5.97	7.7	0.360	4.23
Maxey	September 2007	2.56	7.5	0.180	4.21
Higinbotham	September 2007	0.12	7.4	0.220	5.41
Stevens	September 2007	1.78	7.8	0.140	4.77

Table 4.5. Continued.

Lake	Sampling Date	Sodium (ppm)	Sodium absorption ratio	Sulfate (ppm)	Total dissolved solids (ppm)
Maxey	October 2006	7.22	0.33	13.21	117.12
Higinbotham	October 2006	6.43	0.33	7.86	106.24
Stevens	October 2006	8.02	0.36	11.09	128.00
Maxey	November 2006	19.87	0.83	21.76	136.32
Higinbotham	November 2006	20.51	0.99	16.29	112.64
Stevens	November 2006	196.47	5.17	166.87	764.80
Maxey	December 2006	15.88	0.62	20.78	151.04
Higinbotham	December 2006	11.50	0.49	17.99	147.20
Stevens	December 2006	11.46	0.05	17.30	145.92
Maxey	January 2007	22.91	0.98	15.39	176.64
Higinbotham	January 2007	15.14	0.68	17.08	142.72
Stevens	January 2007	17.95	0.74	12.79	172.16
Maxey	February 2007	61.41	2.36	27.45	270.42
Higinbotham	February 2007	18.87	0.89	12.61	135.04
Stevens	February 2007	37.78	1.51	13.32	215.68
Maxey	March 2007	22.65	1.14	9.66	131.20
Higinbotham	March 2007	6.14	0.34	3.54	79.10
Stevens	March 2007	22.09	1.11	9.42	133.76
Maxey	April 2007	8.31	0.46	5.51	96.64
Higinbotham	April 2007	2.97	0.18	2.16	69.12
Stevens	April 2007	8.51	0.49	2.19	93.44
Maxey	May 2007	5.89	0.30	6.58	88.96
Higinbotham	May 2007	2.39	0.14	2.96	63.36
Stevens	May 2007	8.21	0.39	6.62	131.20
Maxey	June 2007	5.09	0.27	6.83	90.24
Higinbotham	June 2007	2.56	0.15	3.21	69.76
Stevens	June 2007	5.29	0.25	5.30	108.80
Maxey	July 2007	5.66	0.31	7.65	93.44
Higinbotham	July 2007	2.58	0.14	4.09	81.92
Stevens	July 2007	5.66	0.31	7.65	93.44
Maxey	August 2007	20.94	0.78	21.47	165.12
Higinbotham	August 2007	3.40	0.17	4.06	90.24
Stevens	August 2007	4.89	0.25	6.77	96.00
Maxey	September 2007	17.27	0.76	16.33	155.52
Higinbotham	September 2007	5.22	0.29	5.23	88.96
Stevens	September 2007	5.08	0.24	8.26	109.44

Table 4.6. Multiple regression model summary using bicarbonate, boron, calcium, carbonate, chloride, lake, magnesium, nitrate nitrogen, pH, phosphorus, potassium, sampling date, sodium, sulfate, sodium absorption ratio, and total dissolved solids as predictors. Conductivity was excluded from the analysis. The dependent variable was the number of transformants.

R	R^2	Adjusted R^2	Standard error of the estimate	Change statistics					Durbin-Watson
				R^2 Change	F Change	df1	df2	Significant F change	
0.749	0.562	0.193	114.8674	0.562	1.522	16	19	0.190	1.934

Table 4.7. Pearson correlation table and corresponding 1-tailed significance values. Shaded rows and columns indicate correlations with transformation.

	Variable	Date of sampling	Transformation	Bicarbonate	Boron	Calcium	Carbonate	Chloride	Conductivity	Lake	Magnesium	Nitrate	pH	Phosphorous	Potassium	Sodium	Sodium absorption ratio	Sulfate	Total dissolved solids
Pearson correlation coefficients	Date of sampling	1.000	-0.181	0.604	-0.253	-0.383	0.315	-0.311	-0.332	0.000	-0.362	-0.287	0.206	0.336	-0.413	-0.315	-0.336	-0.321	-0.332
	Transformation	-0.181	1.000	*-0.311	-0.040	0.020	-0.143	-0.036	-0.010	-0.097	0.035	-0.122	-0.099	-0.060	0.203	-0.039	-0.022	-0.008	-0.010
	Bicarbonate	0.604	*-0.311	1.000	-0.188	-0.258	0.285	-0.094	-0.181	0.035	-0.258	-0.407	0.334	0.410	-0.130	-0.125	-0.059	-0.270	-0.181
	Boron	-0.253	-0.040	-0.188	1.000	0.729	0.005	0.919	0.926	0.112	0.950	-0.099	-0.193	-0.013	0.562	0.930	0.871	0.957	0.926
	Calcium	-0.383	0.020	-0.258	0.729	1.000	-0.096	0.765	0.854	0.084	0.848	0.115	-0.147	0.184	0.742	0.795	0.773	0.806	0.854
	Carbonate	0.315	-0.143	0.285	0.005	-0.096	1.000	-0.006	-0.047	-0.233	-0.060	-0.291	0.792	0.103	-0.179	-0.025	-0.009	-0.062	-0.047
	Chloride	-0.311	-0.036	-0.094	0.919	0.765	-0.006	1.000	0.974	0.145	0.954	-0.116	-0.150	0.063	0.703	0.985	0.966	0.945	0.974
	Conductivity	-0.332	-0.010	-0.181	0.926	0.854	-0.047	0.974	1.000	0.156	0.986	-0.037	-0.169	0.029	0.730	0.988	0.957	0.975	1.000
	Lake	0.000	-0.097	0.035	0.112	0.084	-0.233	0.145	0.156	1.000	0.083	0.099	-0.278	-0.059	0.302	0.125	0.067	0.122	0.156
	Magnesium	-0.362	0.035	-0.258	0.950	0.848	-0.060	0.954	0.986	0.083	1.000	-0.013	-0.195	-0.001	0.686	0.975	0.933	0.990	0.986
	Nitrate	-0.287	-0.122	-0.407	-0.099	0.115	-0.291	-0.116	-0.037	0.099	-0.013	1.000	0.243	0.060	0.066	-0.074	-0.101	0.017	-0.037
	pH	0.206	-0.099	0.334	-0.193	-0.147	0.792	-0.150	-0.169	-0.278	-0.195	-0.243	1.000	-0.047	-0.233	-0.146	-0.077	-0.218	-0.169
	Phosphorous	0.336	-0.060	0.410	-0.013	0.184	0.103	0.063	0.029	-0.059	-0.001	0.060	-0.047	1.000	0.283	0.031	0.046	-0.029	0.029
	Potassium	-0.413	0.203	-0.130	0.562	0.742	-0.179	0.703	0.730	0.302	0.686	0.066	-0.233	0.283	1.000	0.702	0.722	0.631	0.730
	Sodium	-0.315	-0.039	-0.125	0.930	0.795	-0.025	0.985	0.988	0.125	0.975	-0.074	-0.146	0.031	0.702	1.000	0.980	0.971	0.988
	Sodium absorption ratio	-0.336	-0.022	0.059	0.871	0.773	-0.009	0.966	0.957	0.067	0.933	-0.101	-0.077	0.046	0.722	0.980	1.000	0.913	0.957
	Sulfate	-0.321	-0.008	-0.270	0.957	0.806	-0.062	0.945	0.975	0.122	0.990	0.017	-0.218	-0.029	0.631	0.971	0.913	1.000	0.975
	Total dissolved solids	-0.332	-0.010	-0.181	0.926	0.854	-0.047	0.974	1.000	0.156	0.986	-0.037	-0.169	0.028	0.730	0.988	0.957	0.975	1.000

* indicates significant correlation at the 0.05 level between transformation and the measured component

Table 4.7. Continued.

	Variable	Date of sampling	Transformation	Bicarbonate	Boron	Calcium	Carbonate	Chloride	Conductivity	Lake	Magnesium	Nitrate	pH	Phosphorous	Potassium	Sodium	Sodium absorption ratio	Sulfate	Total dissolved solids
Significance (1-tailed)	Date of sampling	-	0.146	0.000	0.068	0.011	0.031	0.033	0.024	0.500	0.015	0.045	0.114	0.023	0.006	0.031	0.022	0.028	0.024
	Transformation	0.146	-	*0.033	0.409	0.454	0.202	0.418	0.477	0.287	0.420	0.239	0.283	0.363	0.118	0.410	0.448	0.481	0.477
	Bicarbonate	0.000	*0.033	-	0.136	0.064	0.046	0.292	0.145	0.421	0.064	0.007	0.023	0.006	0.225	0.235	0.365	0.055	0.145
	Boron	0.068	0.409	0.136	-	0.000	0.488	0.000	0.000	0.258	0.000	0.282	0.130	0.469	0.000	0.000	0.000	0.000	0.000
	Calcium	0.011	0.454	0.064	0.000	-	0.289	0.000	0.000	0.313	0.000	0.252	0.196	0.142	0.000	0.000	0.000	0.000	0.000
	Carbonate	0.031	0.202	0.046	0.488	0.289	-	0.486	0.393	0.085	0.365	0.042	0.000	0.275	0.148	0.444	0.479	0.361	0.393
	Chloride	0.033	0.418	0.292	0.000	0.000	0.486	-	0.000	0.200	0.000	0.249	0.192	0.359	0.000	0.000	0.000	0.000	0.000
	Conductivity	0.024	0.477	0.145	0.000	0.000	0.393	0.000	-	0.182	0.000	0.416	0.162	0.434	0.000	0.000	0.000	0.000	0.000
	Lake	0.500	0.287	0.421	0.258	0.313	0.085	0.200	0.182	-	0.315	0.283	0.050	0.366	0.037	0.234	0.350	0.239	0.182
	Magnesium	0.015	0.420	0.064	0.000	0.000	0.365	0.000	0.000	0.315	-	0.469	0.127	0.498	0.000	0.000	0.000	0.000	0.000
	Nitrate	0.045	0.239	0.007	0.282	0.252	0.042	0.249	0.416	0.283	0.469	-	0.077	0.363	0.351	0.333	0.279	0.460	0.416
	pH	0.114	0.283	0.023	0.130	0.196	0.000	0.192	0.162	0.050	0.127	0.077	-	0.392	0.085	0.197	0.329	0.101	0.162
	Phosphorous	0.023	0.363	0.006	0.469	0.142	0.275	0.359	0.434	0.366	0.498	0.363	0.392	-	0.047	0.429	0.395	0.434	0.434
	Potassium	0.006	0.118	0.225	0.000	0.000	0.148	0.000	0.000	0.037	0.000	0.351	0.085	0.047	-	0.000	0.000	0.000	0.000
	Sodium	0.031	0.410	0.235	0.000	0.000	0.444	0.000	0.000	0.234	0.000	0.333	0.197	0.429	0.000	-	0.000	0.000	0.000
	Sodium absorption ratio	0.022	0.448	0.365	0.000	0.000	0.479	0.000	0.000	0.350	0.000	0.279	0.329	0.395	0.000	0.000	-	0.000	0.000
	Sulfate	0.028	0.481	0.055	0.000	0.000	0.361	0.000	0.000	0.239	0.000	0.460	0.101	0.434	0.000	0.000	0.000	-	0.000
	Total dissolved solids	0.024	0.477	0.145	0.000	0.000	0.393	0.000	0.000	0.182	0.000	0.416	0.162	0.435	0.000	0.000	0.000	0.000	-

* indicates significant correlation at the 0.05 level between transformation and the measured component

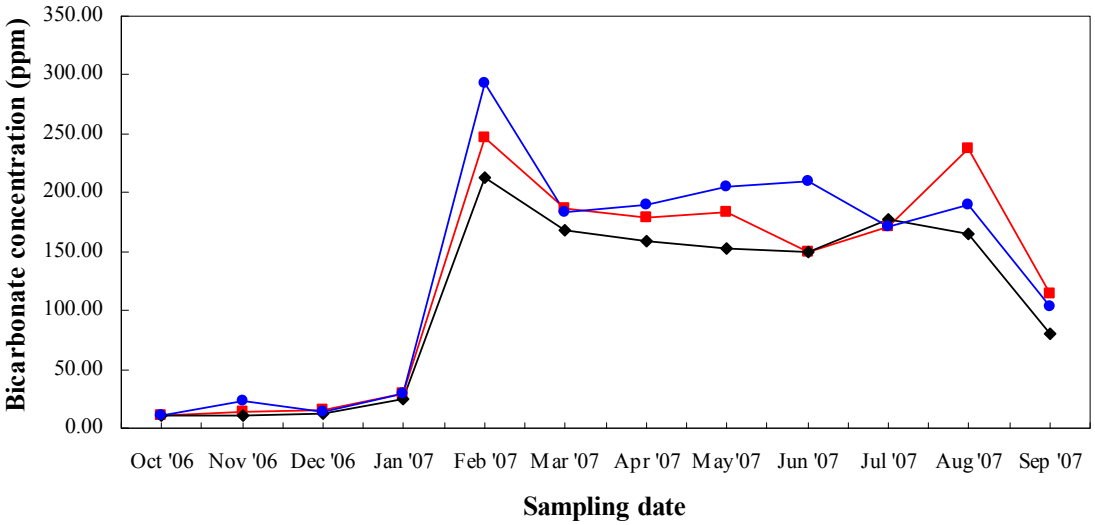

Figure 4.1. Bicarbonate concentrations (ppm) measured for each lake over the 12-month sampling period. Symbols: (♦) Higinbotham, (■) Maxey, (●) Stevens.

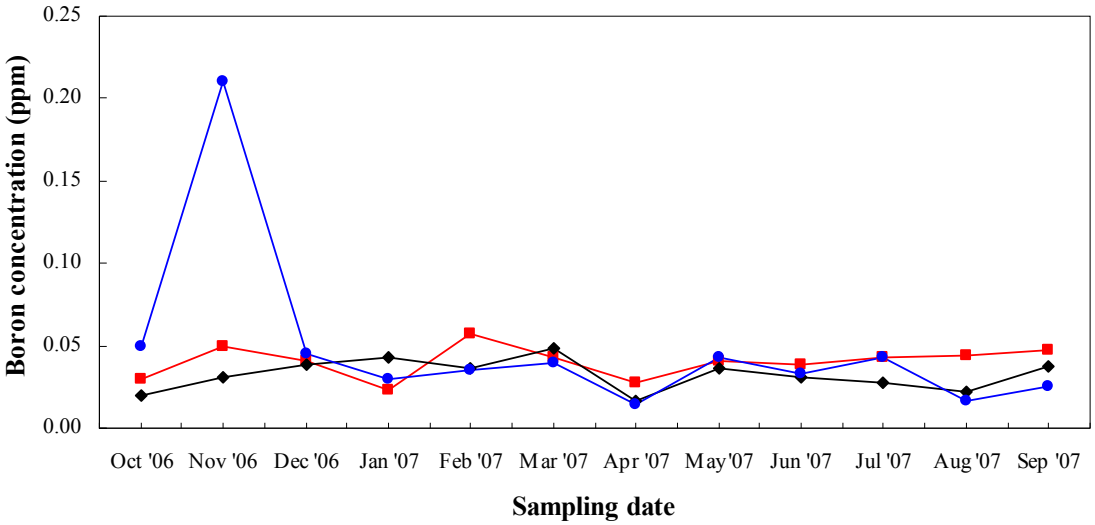

Figure 4.2. Boron concentrations (ppm) measured for each lake over the 12-month sampling period. Symbols: (♦) Higinbotham, (■) Maxey, (●) Stevens.

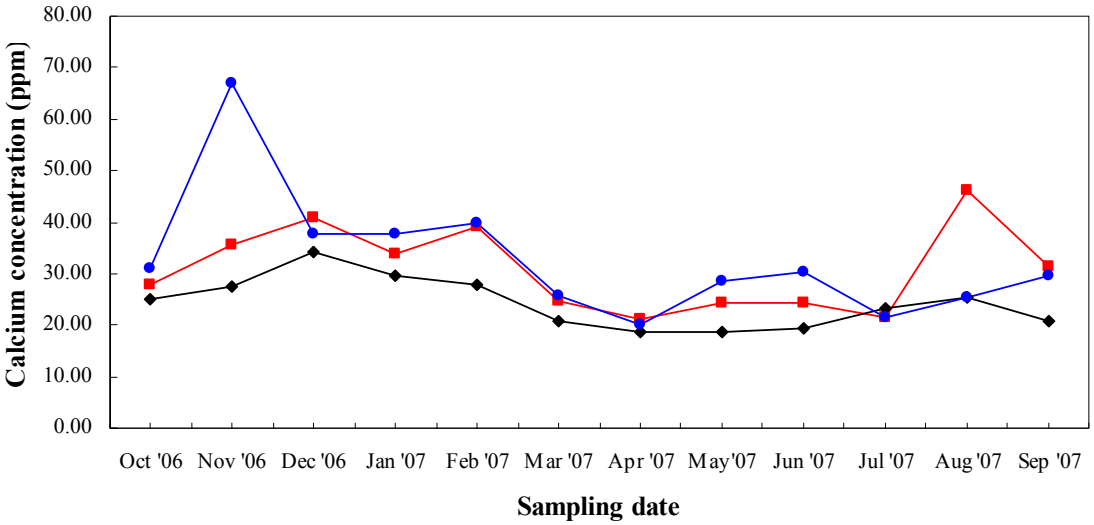

Figure 4.3. Calcium concentrations (ppm) measured for each lake over the 12-month sampling period. Symbols: (♦) Higinbotham, (■) Maxey, (●) Stevens.

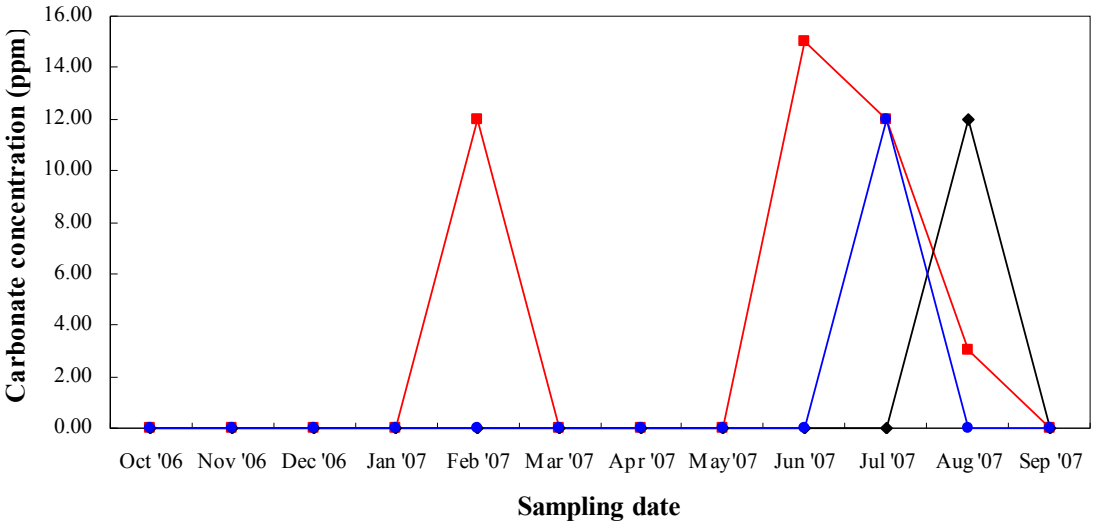

Figure 4.4. Carbonate concentrations (ppm) measured for each lake over the 12-month sampling period. Symbols: (♦) Higinbotham, (■) Maxey, (●) Stevens.

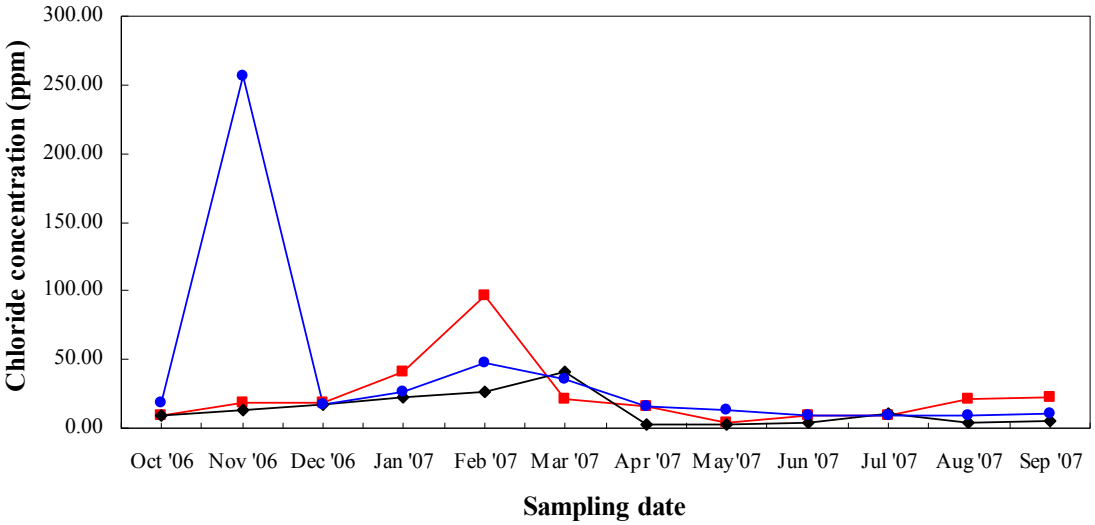

Figure 4.5. Chloride concentrations (ppm) measured for each lake over the 12-month sampling period. Symbols: (♦) Higinbotham, (■) Maxey, (●) Stevens.

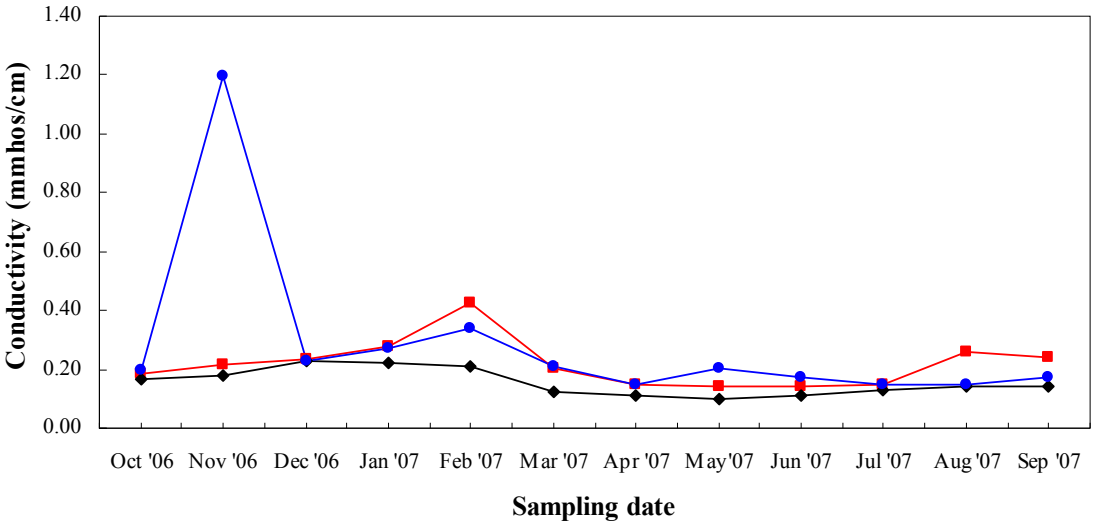

Figure 4.6. Conductivity (mmhos/cm) measured for each lake over the 12-month sampling period. Symbols: (♦) Higinbotham, (■) Maxey, (●) Stevens.

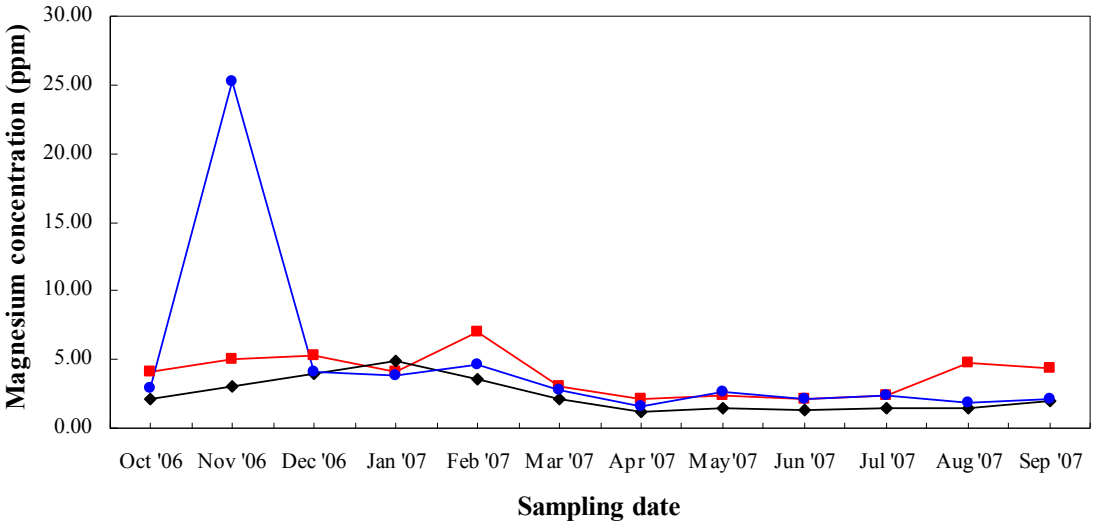

Figure 4.7. Magnesium concentrations (ppm) measured for each lake over the 12-month sampling period. Symbols: (♦) Higinbotham, (■) Maxey, (●) Stevens.

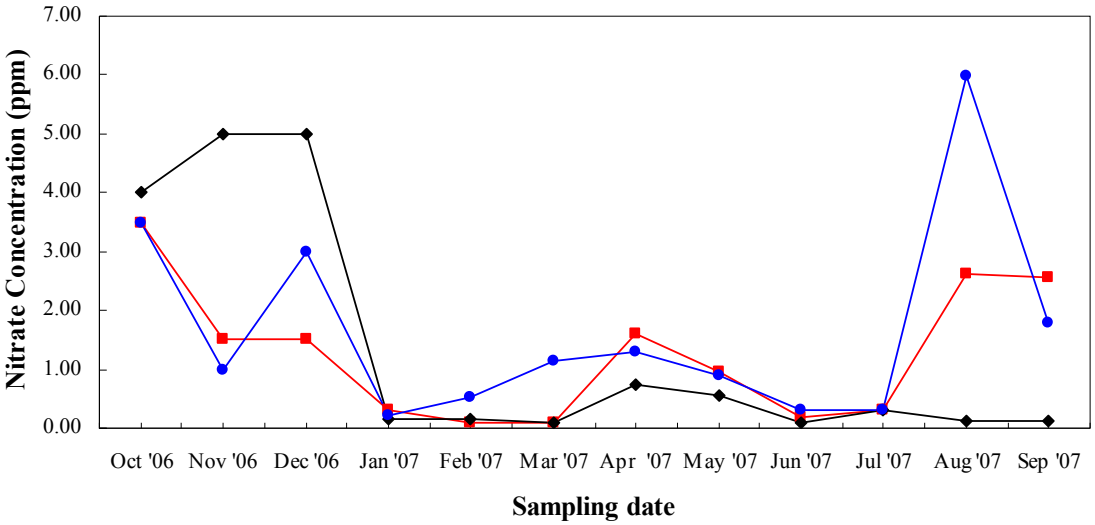

Figure 4.8. Nitrate concentrations (ppm) measured for each lake over the 12-month sampling period. Symbols: (♦) Higinbotham, (■) Maxey, (●) Stevens.

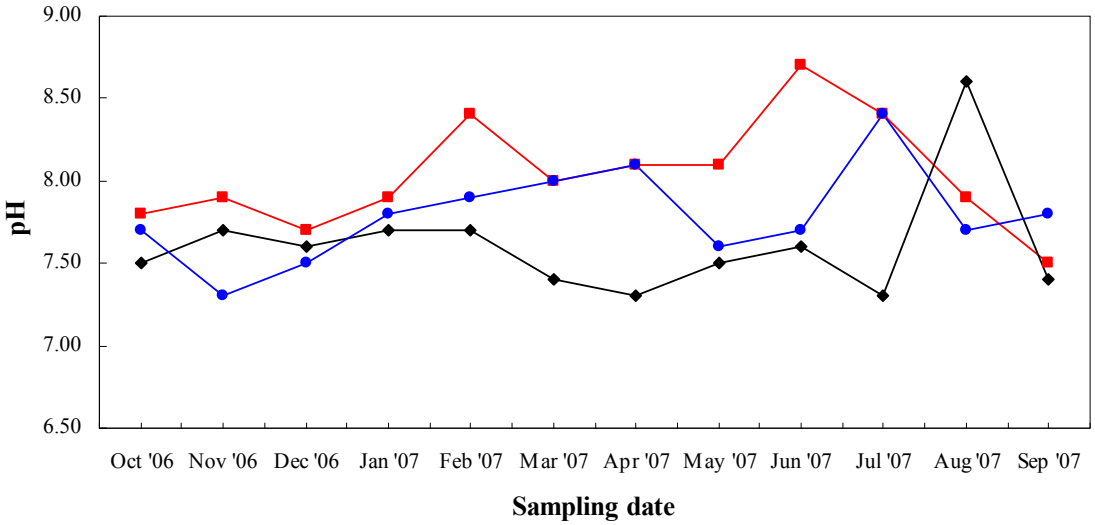

Figure 4.9. pH values measured for each lake over the 12-month sampling period. Symbols: (♦) Higinbotham, (■) Maxey, (●) Stevens.

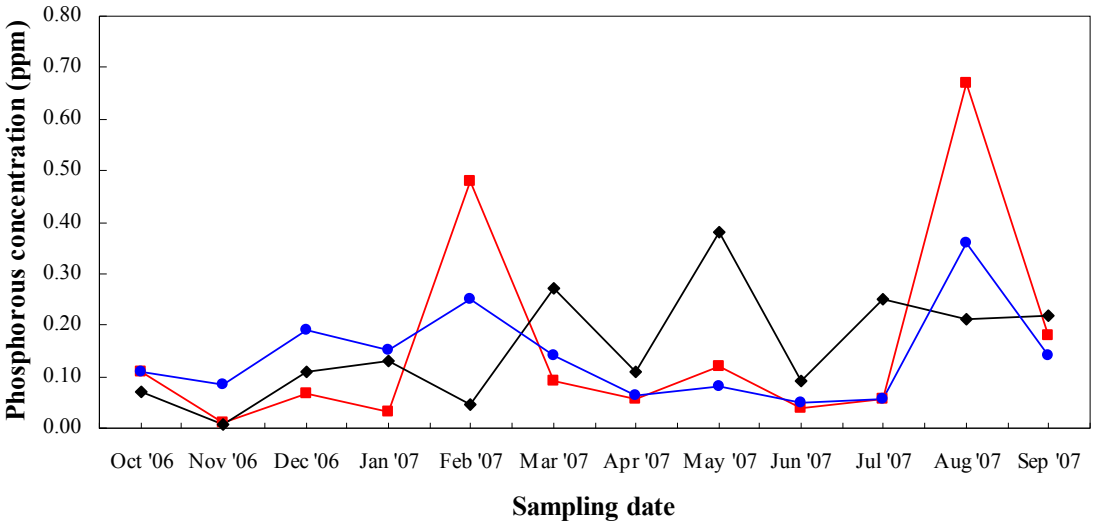

Figure 4.10. Phosphorus concentrations (ppm) measured for each lake over the 12-month sampling period. Symbols: (♦) Higinbotham, (■) Maxey, (●) Stevens.

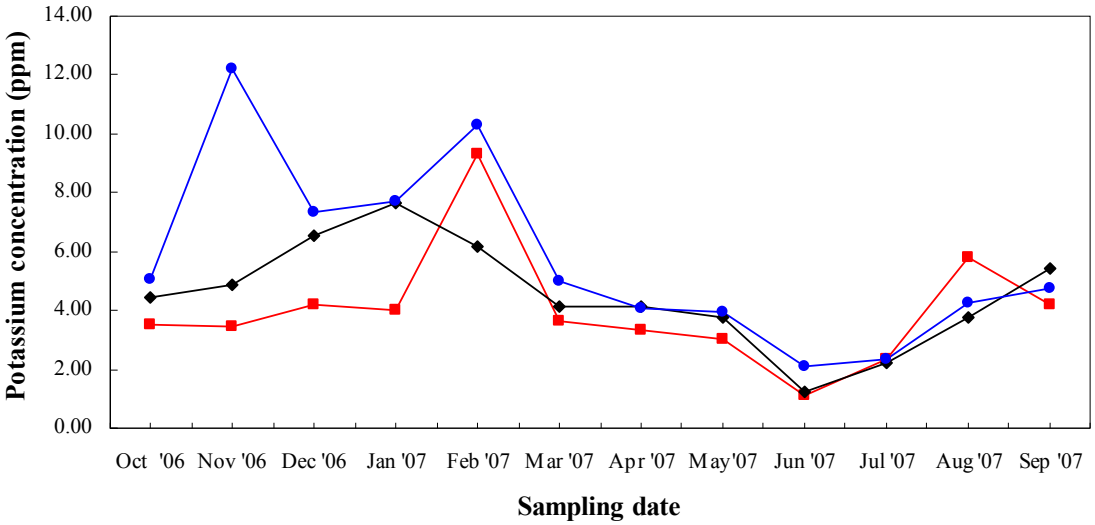

Figure 4.11. Potassium concentrations (ppm) measured for each lake over the 12-month sampling period. Symbols: (♦) Higinbotham, (■) Maxey, (●) Stevens.

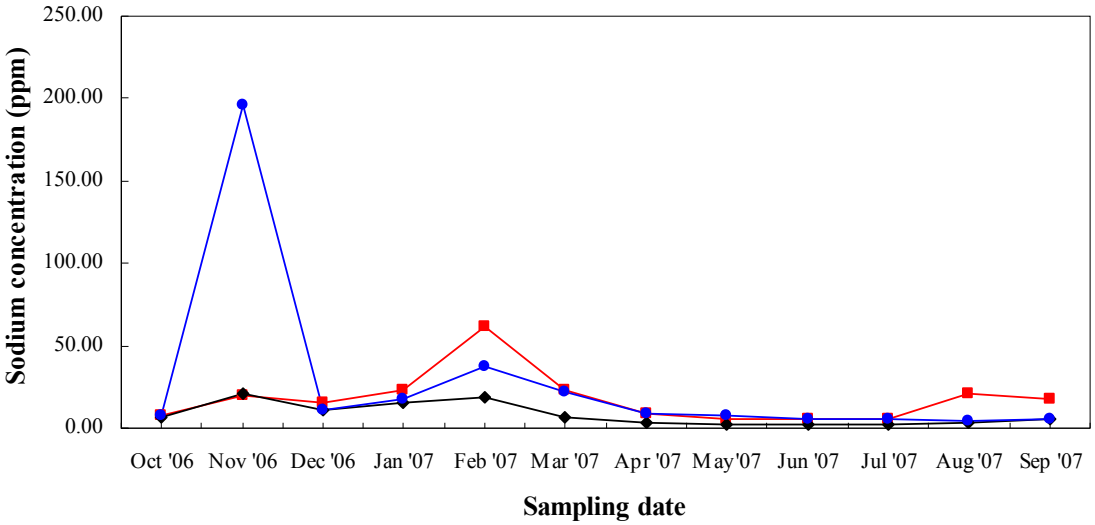

Figure 4.12. Sodium concentrations (ppm) measured for each lake over the 12-month sampling period. Symbols: (♦) Higinbotham, (■) Maxey, (●) Stevens.

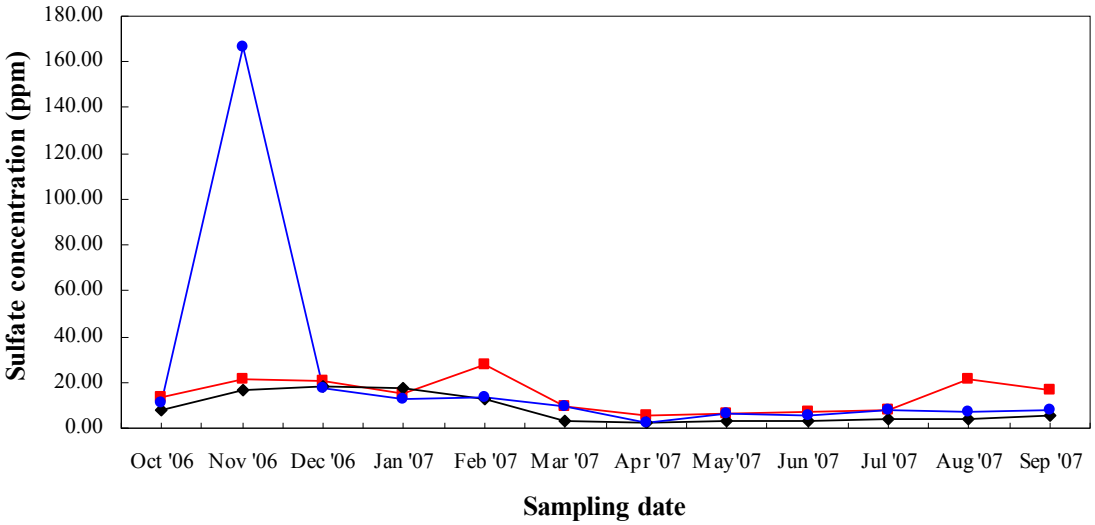

Figure 4.13. Sulfate concentrations (ppm) measured for each lake over the 12-month sampling period. Symbols: (♦) Higinbotham, (■) Maxey, (●) Stevens.

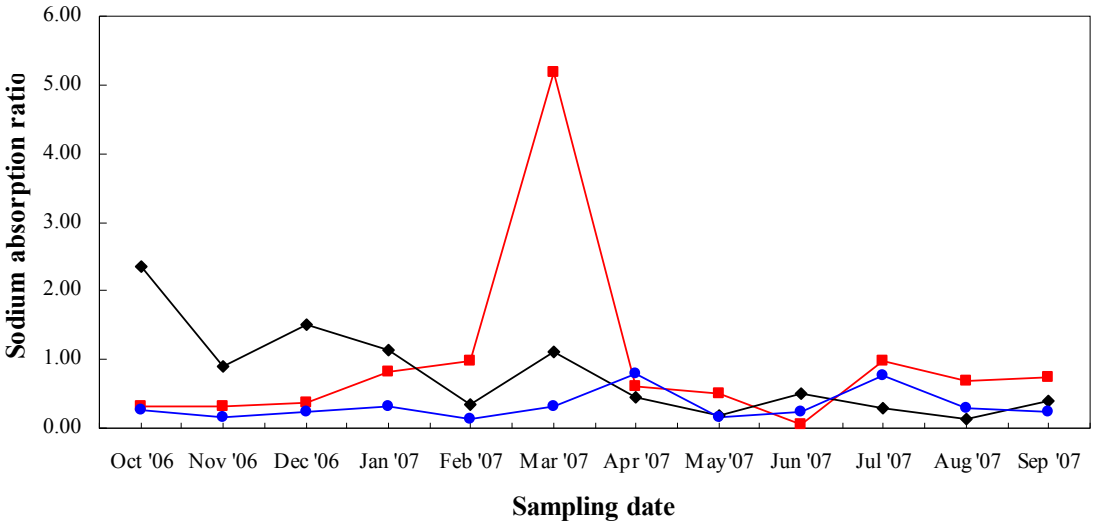

Figure 4.14. Sodium absorption ratios measured for each lake over the 12-month sampling period. Symbols: (♦) Higinbotham, (■) Maxey, (●) Stevens.

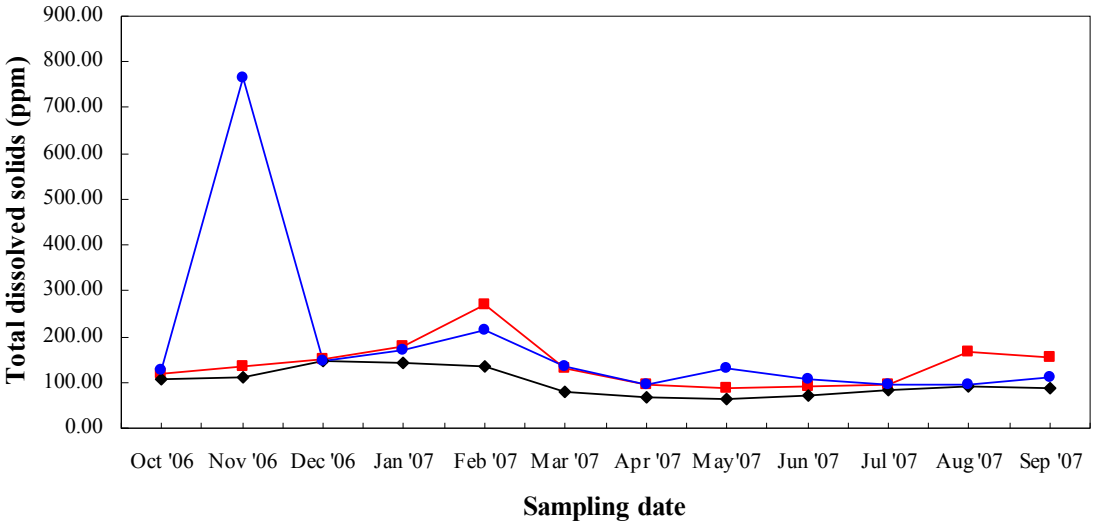

Figure 4.15. Total dissolved solids concentrations (ppm) measured for each lake over the 12-month sampling period. Symbols: (♦) Higinbotham, (■) Maxey, (●) Stevens.

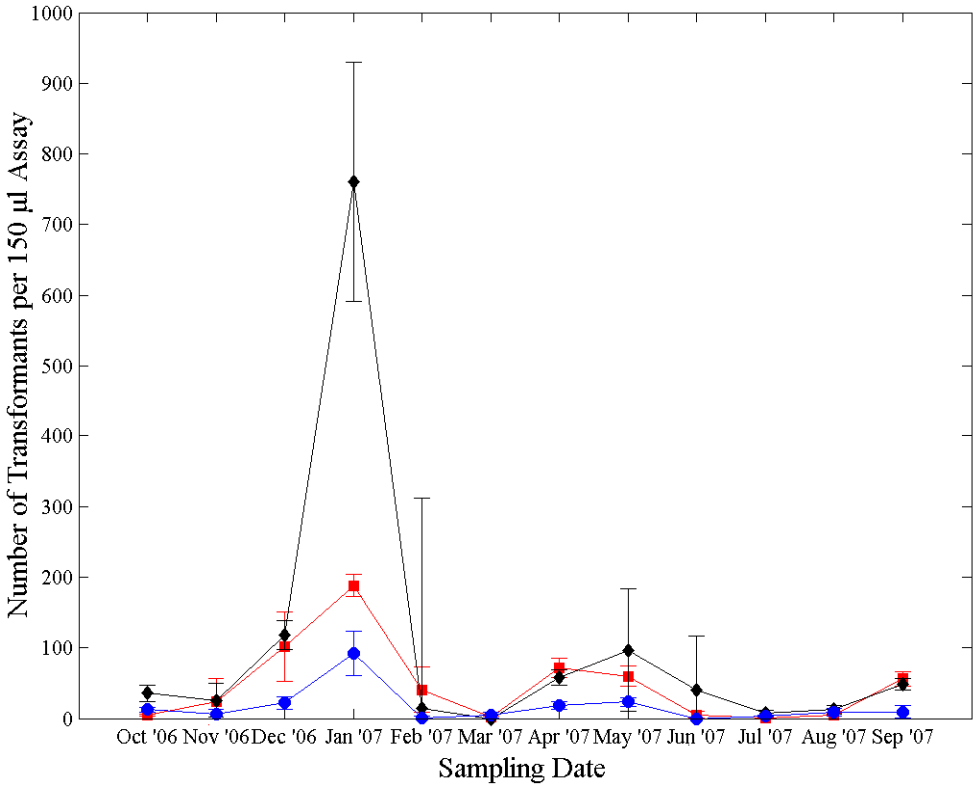

Figure 4.16. Transformation in playa water over time. Symbols: (♦) Higinbotham, (■) Maxey, (●) Stevens.

CHAPTER V

CONCLUDING SUMMARY AND REMARKS

This study has described natural transformation in environmental isolates of *Aeromonas* with regard to competence induction, optimal physiological transformation conditions, DNA concentration and exposure requirements, and four genes essential to the process. Forty-five additional aeromonads were analyzed for their ability to act as donors and recipients with respect to other strains. Finally, transformation was found to occur in water collected from the natural habitats of the species.

A model of *Aeromonas* transformation in the natural environment can be deduced from the results collected. Competence induction in *Aeromonas* is unlike that in any other bacteria in that it takes place in late stationary phase. This indicates that aeromonads are not competent when they are actively growing and nutrients are in abundance. Rather, competence occurs under conditions of nutrient depletion or physiological stress. The finding that the *ptsI* and *clpA/clpS* gene products are required for transformation to occur supports this hypothesis.

The *ptsI* gene product is Enzyme I of the phosphotransferase system. As glucose or other substrates are transported into the cell, a phosphate from phosphoenolpyruvate is transferred to the histidine residue on Enzyme I. Enzyme I then transfers the phosphate to histidine protein, which in turn transfers the phosphate to Enzyme II. Enzyme II then transfers the phosphate to glucose, forming glucose-6-phosphate. When glucose concentrations are low Enzyme II accumulates in the cell and activates adenylyl cyclase

causing cyclic AMP levels to rise. This cyclic AMP activates cAMP-cAMP receptor protein (Crp) which binds to the promoters of DNA, allowing the transcription of genes needed under nutrient-limiting conditions. Without Enzyme I, Crp cannot be activated so necessary genes cannot be transcribed. It is proposed that this cascade of molecular interactions occurs under nutrient-limiting conditions allowing for aeromonad competence genes and different sigma factors to be transcribed. ClpA/ClpS could also play a role in the regulation of transcription of competence genes under stress conditions as has been shown in other competent bacteria with other Clp proteins.

Furthermore, competence in *Aeromonas* is sustained over hours, days, and possibly even longer. Competence in *Aeromonas* is not constitutive in the strictest sense, but the cells in the natural environment may spend most, if not all, of their lives in a state of competency due to low nutrients and less than "perfect" growth conditions. Induction and maintenance of competence could be due to a stationary phase sigma factor inducing the transcription of competence genes at this time.

When in the natural low-nutrient environment of a playa lake, the cells are most likely competent, and the chemical conditions of the water are appropriate for a low level of transformation. These conditions are suboptimal when compared to laboratory conditions, but there is enough sodium and calcium or magnesium to facilitate the process. Even a low level of transformation among some isolates can have consequences for the aeromonad population as a whole. Horizontal gene flow can be the mechanism by which genetic variability is introduced into populations of aeromonads. Generally, transformation occurs between closely related strains, but there are also a minority of

strains that are broadly transformable that can take up, integrate, and express DNA from more distantly related strains in different *Aeromonas* species. Rarely are competent bacteria transformable with DNA from other species within the same genus as some strains of *Aeromonas* are. If a beneficial gene were to be introduced into the population, it could be disseminated through the population much more quickly through natural transformation and horizontal gene transfer then through vertical gene transfer. Conversely, there is a sub-population of aeromonads in each transformation group that are non-competent. They cannot directly benefit from advantageous genes obtained from transformation. However, these strains can serve as a reservoir of functional aeromonad genes to "protect" the population from deleterious genes or mutations obtained through horizontal gene transfer.

This research has laid the foundation for developing this model for transformation in *Aeromonas* and improving models for other environmental bacteria. More research should be done in order to fully describe the genes already identified and other necessary genes and their molecular roles in the development of competence and DNA binding, uptake, integration, and expression. It would also be of value to know what other factors affect transformation in the natural environment. This research has assessed transformation capability of aeromonads only in surface water from the playa lakes. It is unknown where the majority of the aeromonad population resides in bodies of water. In biofilms, on rocks, or on fish? In deeper locations within the water column? In association with sediments? Aeromonads have been isolated from all of these locations, but it is unknown if aeromonad transformation is occurring in these habitats and at what

levels. The answers to these questions along with the data collected in this study will allow a deeper understanding of the interactions between the environment, genetics, and evolution of aeromonads.