Introduction

Bacteria in the genus *Aeromonas* are ubiquitous in aquatic environments. Aeromonads are also known to cause opportunistic infections and self-limiting gastrointestinal infections. Sakai (year) and Huddleston (2013) reported that *Aeromonas salmonicida* is capable of achieving competence and undergoing natural transformation.

Natural transformation is a form of horizontal gene transfer. In this process a bacterial cell takes up free DNA from the environment to use for its own purposes which is typically the expression of a new gene but could also include protection against bacteriophages, nutrient exploitation, regulation of gene expression, or DNA repair (Lorenz and Wackernagel, 1994). Natural transformation can be thought of as a six-part process beginning with competence induction, DNA binding, and fragmentation, and ending with uptake, integration and expression of the transformed DNA sequences (Huddleston, 2008).

However, before natural transformation can occur, there must be an appropriate environment. Free DNA is continuously produced by cellular lysis and excretion. Therefore, transformation by free DNA is more likely to occur in habitats with high bacterial concentrations, which includes the environment existing in the intestines of insects, worms, and warm-blooded animals (Lorenz and Wackernagel, 1994). In the human large intestine, the levels of horizontal gene transfer cannot be detected because of the cell numbers and diversity of microbes present (Huddleston, 2014). In particular, natural transformation in this habitat is underestimated.

Many antibiotics work through the stomach and intestines. Antibiotics create an environment in which antibiotic resistant populations are selected. These antibiotic resistance populations then expand clonally and through horizontal transfer of these resistance genes (Huddleston, 2014).

Considering that antibiotics can cause enough stress to induce competence and that the environment of the human gastrointestinal system is conducive for natural transformation (Huddleston, 2014), the pathogenic tendencies of *Aeromonas* give rise to the threat of antibiotic resistance. In fact, both environmental and clinical aeromonad isolates have demonstrated resistance to antimicrobial agents, thereby making these *Aeromonas* infections more difficult to treat (Huddleston et al., 2013).

While *Aeromonas* has the ability of natural transformation not all bacteria do. For this capability, a bacterium requires the participation of certain cellular functions that are provided by genes located throughout the bacterial chromosome and that may only be expressed under particular environmental conditions (Lorenz and Wackernagel, 1994). In order for a state of competence to be achieved, genes whose proteins provide these necessary functions must be expressed (Lorenz and Wackernagel, 1994).

Naturally occurring conditions that could induce natural transformation may include a decrease in temperature, the presence of solutions of certain electrolytes, and the presence of lysozymes and proteolytic enzymes (Lorenz and Wackernagel, 1994). The competence inducing conditions for the bacteria of *Aeromonas* are known and include nutrient-limiting conditions and being in the stationary phase of

growth (Huddleston, 2008). While genes involved in the natural transformation process have been identified in some genera, few have been identified in this genus. Through random mutagenesis, Huddleston discovered four genes that are necessary for natural transformation in *Aeromonas: tapY1, ptsI, recBC,* and *clpA/clpS* (Huddleston, 2008). In this paper, I will relate my investigation into purpose of the gene *clp*A in the natural transformation ability of *Aeromonas salmonicida*.

ClpA is an ATP-dependent molecular chaperone and the regulatory component of the ClpAP protease (Pak, et al., 1999). It remodels bacteriophage P1 RepA dimers into monomers (Pak and Wickner, 1997). However, when in complex with the protease ClpP, ClpA serves to target specific proteins for degradation by ClpP although it is still capable of its chaperone ability while in this complex (Pak et al., 1999). Since Clp proteins refold and remodel and degrade proteins damaged under stress conditions (Huddleston, 2008), it is reasonable to conclude that there could be an increase in the presence or activity of ClpA at the time of natural transformation.

To test for the importance of ClpA in the natural transformation ability of *Aeromonas salmonicida*, we constructed a mutant fragment containing a gentamycin resistance cassette to replace *clp*A in the wild-type *Aeromonas* genome. After this gene replacement, we would then proceed to conduct tests to compare the wild-type to the mutant *Aeromonas* strain and determine if there is a difference in their transformation abilities.

Methods

PCR amplification. Using the previously extracted DNA from *Aeromonas salmonicida* strain #92, approximately 500 base pairs from the downstream and upstream *clp*A region were amplified through Polymerase Chain Reaction (PCR) using the designed primers indicated in the table below.

The gentamycin resistance cassette (Gm^R) was amplified through PCR from *Aeromonas* strain #771 which has been engineered to contain the gene for gentamycin resistance (Gm^R). The primers used are indicated in Table 1.

Table 1. PCR products and primers used to construct the *clpA* replacement fragment. The underlined sequences indicate homology with the Gm^R cassette.

Product	Upstream Primer	Downstream Primer	Product size
Down-stream *clp*A	JRH0021: 5′- GCAAGATGAATTACAAGCGCC-3′	JRH0022: 5′- CCAAGTACCGCCACCTAAG CACTGATAGTTAGAAAGCGAC-3′	441
Up-stream *clp*A	JRH0024: 5′- GTTGCTGCTGCGTAACATAGGCACCTCCCCTAGT AC-3′	JRH0025: 5′- TGAGCGAGTCAAGGAGTTG-3′	480
Gm^R	JRH0011: 5′- ATGTTACGCAGCAGCAAC-3′	JRH0010: 5′- TTAGGTGGCGGTACTTGG-3′	526

These were 50 µl PCR reactions that contained 40.75 µl nuclease-free water, 5 µl ThemoPol Reaction Buffer (10X), 1 µl of 10 µM Deoxynucleotide (dNTP) Solution Mix, 1 µl of the indicated DNA template, 0.25 µl VENT DNA Polymerase, and 1 µl of 10 µM of each of the indicated primers. The Thermo Cycler cycling parameters for each reaction were as follows: 1 cycle of 95 °C for 1 minute; 30 cycles of 95 °C for 30 seconds, 56 °C for 30 seconds, 72 °C for 30 seconds; followed by 1 cycle of 72 °C for 10 minutes, and a hold at 12 °C.

These PCR products were then purified by using the Zymo Research DNA Clean & Concentrator™-25 kit (Irvine, CA). This required adding 250 µl of DNA binding buffer to the 50 µl of PCR product and mixing it in a 1.5 mL microcentrifuge tube before transferring the mixtures to Zymo-Spin™ Columns in Collection tubes. The products underwent microcentrifugation at 16,000 x g for 30 seconds. The flow-through was discarded before 200µl of DNA Wash Buffer was added to each column and microcentrifuged at 16,000 x g for 30 seconds. This step was repeated once before transferring the Zymo-Spin™ Columns to 1.5 microcentrifuge tubes. Then 30 µl of DNA elution buffer was added directly to column and was allowed to incubate at room temperature for 1 minute before centrifugation at 16,000 x g for 30 seconds. At this point, the 1.5 microcentrifuge tubes containing the purified fragments were labeled and stored in -20 °C conditions.

Constructing the mutant fragment. Splicing overlap extension polymerase chain reaction (SOE-PCR) was used to combine the three amplified pieces. First the gentamycin resistance cassette and the upstream *clp*A region were combined using this method. Then the downstream *clp*A region was attached using SOE-PCR followed by a gel extraction.

The 50 µl SOE-PCR reaction used to combine the Gm^R and the upstream *clp*A region contained 40.75 µl nuclease-free water, 5 µl ThemoPol Reaction Buffer (10X), 1 µl of 10 µM Deoxynucleotide (dNTP) Solution Mix, 0.5 µl of the amplified Gm^R, 0.5 µl of the amplified upstream *clp*A region, 0.25 µl VENT DNA Polymerase, and 1 µl of 10 µM of each of the primers JRH0025 and JRH0010.