Identification of a putative two-component gold-sensor histidine kinase regulator in *Stenotrophomonas maltophilia* OR02

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ABSTRACT

Stenotrophomonas maltophilia OR02 (*S. maltophilia* 02) is a multi-metal resistant bacterium that was obtained from a heavy-metal contaminated site in Oak Ridge, TN. This location contains electromagnetic isotope processing centers that generated deleterious waste that was released into the surrounding environment in the form of mercury, uranium nitrate, and extensive heavy metals amalgamations. To survive under such conditions, metal resistant bacteria encode proteins that alter the toxicity of the metals in oxidation/reduction reactions, sequester them, or pump them out of the cell entirely.

A gold-sensitive mutant was generated by introducing the EZ-Tn5™ transposome into *S. maltophilia* 02. This transposon, which carried a kanamycin resistance gene, randomly incorporated itself into the *S. maltophilia* 02 genome. 880 transformants were replica plated onto LB-kan plates, revealing a gold-sensitive *S. maltophilia* 02 mutant, A12. Liquid culture experiments showed that the minimal inhibitory concentration (MIC) for A12 was 170 μM gold chloride, whereas the wild type *S. maltophilia* 02 grew well into high concentrations of gold salts up to 190 μM. DNA sequencing and Basic Local Alignment Search Tool (BLAST) analysis showed that the transposon inserted itself after a hypothetical protein and downstream of genes for a DNA binding protein and a two-component sensor histidine kinase. BLAST analysis revealed a sequence similar to the sensor protein PhoQ, one component of a two-component histidine kinase.

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Table of Contents

CHAPTER I: INTRODUCTION

1.1 Background (Y-12 Plant) and East Fork Poplar Creek

The Y-12 Plant's purpose was to enrich uranium through electromagnetic separation, followed by gaseous diffusion, for the manufacturing of nuclear weapons components. The entire Y-12 National Security Complex was a contributor site to the original Manhattan Project, processing and creating enriched uranium for the "Little Boy" bomb dropped on Hiroshima in 1945 [13]. The site is named from the World War II code name for the electromagnetic isotope separation plant based in Clinton Engineer Works at Oak Ridge, TN. Oak Ridge, Tennessee remains the site of facilities for development of nuclear weapons. Uranium and other heavy metals were dumped into the surrounding watershed drained by East Fork Poplar Creek (Poplar Creek). Poplar Creek originates from a spring underlying the Y-12 plant and is initially confined to a man-made canal which flows through the Y-12 plant [3,13]. State and federal agencies are still funding the removal of these contaminates in the areas surrounding the Y-12 plant, although the environment still maintains high levels of pollutants.

Four waste confining S-3 ponds, located near the Y-12 plant at the origin of Poplar Creek, were used to dispose uranium and heavy metal contaminated acid washes [4]. These ponds were constructed lacking covering or lining to for hopeful evaporation and decontamination of liquid wastes as they passed through the soil. In practice however, most of the wastes simply leached into the ground and the nearby creek and remaining water bodies. The use of these ponds was discontinued in 1983, and remaining contents

were treated while the liquid was drained with subsequent filling and capping of the ponds.

1.2 Stenotrophomonas maltophilia Oak Ridge strain 02

Stenotrophomonas maltophilia Oak Ridge strain 02 (*S. maltophilia* 02) was isolated from East Fork Polar Creek (95). It is an aerobic, non-fermentative, gram negative bacilli. Some strains of this organism are human opportunistic pathogens that cause respiratory and urinary tract infections. Medical therapy is difficult because it has shown resistance to various antibiotics (15). When compared to the multi-metal resistant bacteria, *Enterobacter sp.* YSU, *S. maltophilia* 02 has been experimentally concluded to be more resistant to metals, growing well in the presence of toxic levels of metals such as mercury, gold, copper, selenite, cadmium, lead, chromium, and selenium (14). Its ability to survive in the presence of toxic metal is due to its ability to use different resistance mechanism like oxidation/reduction (redox), efflux or sequestration.

1.3 Xanthomonadales

Xanthomonadales are early divergents of bacteria within *Gammaproteobacteria*. They are one of the largest groups of bacterial phytopathogens. These bacteria negatively affect agriculturally important plants including tomatoes, rice, citrus plants, bananas, and coffee (33). Many species within the order are also pathogenic to humans. Species within the genus *Stenotrophomonas* are opportunistic pathogens with inherent multidrug resistant that are responsible for nosocomial infections in immunodeficient patients (32). *Xanthomonadales* are Gram-negative, catalase positive, non-spore forming

obligate aerobic organisms. Members belonging to the order are straight rods lacking prosthecea. Members can be motile with flagellum or non-motile lacking known motility mechanisms, *Stenotrophomonas* is the only known genus within *Xanthomonadales* able to perform nitrate reduction within the *Xanthomonadaceae* family (32).

1.4 Properties of Gold

Metallic gold is one of the least chemically active metals; it does not oxidize or burn in air when heated and is inert to strong alkalis and nearly all acids, deserving of its title as a "noble metal" (19). Gold constitutes a third of group IB of transmission metals, along with copper and silver. Electronic configuration is comprised of a filled *d* electron shell with a one *s*-shell orbital electron. This, along with the large size (166 pm) bestows great ionization potential and electron affinity. Seven total oxidation states for gold exist, but the relevant forms existing in nature are Au^{+1} and Au^{+3} (18). All members of group IB form stable complexes with ligands containing available electron pairs (Lewis acid-base pairs), which commonly includes sulfur and nitrogen containing groups. These Lewis acid-base reactions are important for its antimicrobial properties (22,27).

1.5 Uses of Gold

Gold conducts electricity, does not tarnish, is readily malleable, can be drawn into wire, thin sheets, alloys with many other metals, and has a wonderful color and luster (35). Almost every established culture in history has used gold to symbolize power, beauty, purity, and accomplishment. Within the United States, jewelry accounts for nearly 40% of gold's allocation and nearly 35% for use in the electronics markets. While ornamental

qualities represent such desire for gold in jewelry, certain chemical properties of gold confer a growing use for more technological applications in modern day. Computational components such as edge connectors, memory chips and plug-and-socket connectors all benefit from gold's ability to yield low current continuously without tarnishing. Gold alloys are used in dentistry because it is chemically inert, nonallergenic, and easy for the dentist to work. Medically, gold has been used in surgical tools, life support equipment, treatment of rheumatoid arthritis, and even specialized treatments against certain cancers. Certain parts of aeronautical equipment like mechanical joints also contain gold due to its resistance to radiation. Although in its infancy, brain machine interface (BMI) technology which functions to treat neurological disorders utilizes gold electrode probes implanted directly into the cerebrum (34).

1.6 Environmental Heavy Metal Pollution

Industrial mining operations require the use of a vast array of machinery and chemical additives to efficiently extract materials from the environment [13]. Although legally regulated, much of these operations seep wastes and reagents from the extraction procedures back into the environment, affecting the ecosystem on some level. Common pollutants include heavy metals such as mercury, cyanide sulfuric acid, ammonium nitrate and saturated hydrocarbons used in the blasting process. Cyanide amalgamation is used for over 90% of modern gold mining applications. Mining requires considerable water usage, with this water eventually leeching back into community water sources that is utilized for agriculture and drinking water. Surface and ground water will remain contaminated for many years after the mining operation ends [7]

Gold concentrations in naturally occurring bodies of water ranges from 1-5 ppt (1-5 ng/L), and typically exist in the Au^{+2} standard observed on the periodic table since Au^{+1} and Au^{+3} redox reaction potential exceeds that of water in unliganded gold, which is a thermodynamic explanation for any aqueous media being an unfavorable environment for common existing gold species [13,7]. This insolubility puts more pressure for gold to wash up on soils and physical surfaces, where bioavailability is greatly increased. Metal deposits and associated industrial setup with their wastes ejected into the surroundings put tremendous environmental pressures on microorganisms living in these contaminated sites.

1.7 Gold Toxicity

Worthwhile discussion on heavy metal toxicity must be defined by whether we are talking about essential heavy metals or non-essential heavy metals. Cu, Cr, Co, and Ni all act as micronutrients and are critical in redox processes. They confer electrostatic interactions to stabilize molecules, manage osmotic pressure, and serve as regular cofactors for enzymes and electron transport chains. Irregular concentrations of these metals are not thought to be as toxic as high concentrations of non-essential heavy metals such as Ag, As, Cd, Hg and Cd. Non-essential heavy metals serve no known biologically important functions, making them more toxic when found in the ecosystem [28,29].

The presence of unintended heavy metals puts incredible pressure on microbial species to develop resistance mechanisms or be circumstantially killed from their location. Bacteria

that are subject to environmental circumstances where toxic amounts of heavy metals are driven to develop internal mechanisms to control the exposure to heavy metals. While certain acidophilic or chemolithotrophic bacteria are inherently useful for extracting metals and other substances through biomining/bioremediation from the environment, *Stenotrophomonas maltophilia* is not one of them [13].

More recently, Au has been detailed as an efficient cofactor for a nicotinamide adenine dinucleotide oxidase in the *Micrococcus luteus*, a gram positive actinomycete (6). Furthermore, the *Cyanobacteria Plectonema boryanum* gains metabolic energy by utilizing Au^{+3}-containing complexes (review for intracellular gold precipitation of cubic gold nanoparticles (38).

Under normal concentrations found in nature, gold serves little toxic threat to humans. Gold can be found in trace amounts in the human body, relegated to hair skin, and nails. Gold (I) is the most common state found in the human body, as increasingly oxidized species like gold (III) complexes undergo high hydrolysis rates and have notable reduction potential under physiological conditions. Gold remains non-essential in the human body, and while we are not considerably harmed by it, microorganisms suffer due to the previously mentioned reduction potential in physiological conditions. The stability of gold-incorporating drugs under physiological condition still remains challenging for developing effective therapeutic agents.

The most broad and conclusive clinical data on biodistribution has been from chrysotherapy (gold therapy and aurotherapy), a process which uses gold salts to stabilize lysosomes, preventing them from damaging bone, cartilage, and connective tissue, which are normally unresponsive to salicylates or nonsteroidal anti-inflammatory agents [19]. The specific gold species gold thiomaleate, gold thioglucose and gold thiosulfate are some of the only known pharmacologic compounds able to bring remission to rheumatoid arthritis and others such as psoriatic arthritis and pemphigus vulgaris.

1.8 Bacteria and Heavy Metal Interaction

Heavy metals such as zinc, cobalt, selenium, copper, and iron are essential for most biological systems. Due to the microscopic size of bacteria, they have a high surface-to-volume ratio, affording a large contact area for continual interaction with their surrounding environment. Bacteria normally possess a negative charge to their cell membrane, which makes them especially susceptible to interactions with heavy metals, further stipulated by whether the cell has ability to metabolize them (13,6).

Among the large diversity of resistance mechanisms found in prokaryotes, transmembrane efflux pumps for extruding cations are well described. Some instances include resistance-nodulation-cell division (RND) superfamily proteins, which form complexes with (i) outer membrane factors (OMF) or (ii) membrane fusion proteins. Both of these complexes are involved in transport of heavy metal ions from the inner cytoplasm to the periplasm, or across the outer membrane from the periplasm to the external environment [31]. An applicable example is the CzcCBA system which mediates

an efflux of Zn^{2+}, Co^{2} and Cd^{2+} ions [31]. P-type ATPases are another family of heavy metal transport proteins involved in both importing inorganic cations from the periplasm to the cytoplasm or outside of the cell, and export of these ions into or out of the cytoplasm. This mechanism is dependent on ATP hydrolysis. Heavy metal substrates for this system that are reported include Ag^{+}, Cu^{2+}, Cd^{2+} and Zn^{2+}. Besides ion transport, detoxification of heavy metal ions is also essential for bacteria survival. Reduction of pentavalent arsenate [As^{+5}] ion to its trivalent arsenite (As^{+3}) vis-à-vis by the arsenate reductase ArsC enables the detoxification and efflux of As(III) through its associated membrane pump protein ArsB [13]. These systems are additionally often regulated in response to the toxic metal ions present, which proceeds to activate transcription of subsequent resistance determinants. The regulator, ArsR, for example induces expression of the *ars* (arsenite/antimonite) resistance operon upon exposure to these ions [14]. CzcD, a cation diffusion facilitator protein is another resistant determinant that partially regulates the expression of the *czcCBA* system [11,36].

Common strategies for the elimination of gold for bacteria and archaea are currently understood to be of two possible mechanisms. The first is the utilization of active efflux of gold ions through metal transporters, such as the Au-resistance *gol* cluster present in *Salmonella*. This is the most well understood mechanism for bacterial heavy metal resistance. (6) The second involves active reduction and eventual precipitation of gold complexes from both cytoplasm and the cellular surface of the reducing bacteria. These secreted metabolites that solubilize gold by forming complexes include thiosulphate cyanide, and certain amino acids [6].

1.9 Salmonella Gold Resistance: CpxR/CpxA System

Very few studies have been performed on mechanisms for gold-specific resistance on bacteria, fungi, or protists, which these mostly focus on general heavy metal resistance pathways (5). Within *Salmonella*, the metalloregulator GolS mediates the response to Au(I) ions for control of the expression of the RND-efflux pump GesABC, among other genes. CpxR/CpxA, a response regulator for a two-component regulatory system via a main cell-envelope stress-responding system, promotes transcription of *gesABC* in the presence of Au(I) ions at neutral pH, and that Au subsequently triggering activation of the CpxR/CpxA system. This coordinated action of GolS and CpxR/CpxA in *S. enterica* is crucial for the survival against Au(I) damage. Removal of the gene and its transcription products leads to the death of the cell at even low concentrations of gold.

1.10 Identification of gold-resistance genes using Transposon Mutagenesis

The EZ-Tn*5*™ <*R6Kγ ori*/KAN-2>Tnp Transposome™ has been used to identify functional genes in bacteria (37). The transposon is a 2000 bp segment with a *R6Kγ* replication origin and a kanamycin resistance gene flanked on each side by 19 bp mosaic ends (ME) which bind transposase. The transposome is the transposon plus transposase bound to each mosaic ends. When introduced into *S. maltophilia* 02, it inserts itself into the genome, rendering it resistant to kanamycin. Transformants are then screened for mutations by replica plating. Tentative identification of the mutated gene involves cloning and DNA sequencing. Genomic DNA from the mutant is digested with enzymes that cut outside the transposon, ligated and transformed into *E. coli* strain ECD100D

pir116. Transformants that grow on kanamycin plates will possess a recombinant plasmid that contained the transposon flanked on each end by a segment of the interrupted chromosomal DNA. The *pir116* gene is required for the *R6Kγ* replication origin to replicate the recombinant plasmid. Sequencing of the interrupted chromosomal DNA provides a tentative identity of the mutated gene. This technique was used to isolate a gold-sensitive, *S. maltophilia* 02 mutant named A12.

Chapter II: Hypothesis

From previous studies on the gold resistance in *Salmonella*, the interrupted gene in A12 is probably related to protein involved in efflux, sequestration and detoxification. In *Salmonella*, the response to Au(I) ions is controlled by the specific metalloregulator GolS, which controls the expression of the RND-efflux pump GesABC, among other genes. CpxR/CpxA, a response regulator for a two-component regulatory system, promotes *gesABC* transcription in the presence of Au ions. Au will also initiate activation of the CpxR/CpxA system. I postulate that the transposon inserted itself into a region of DNA that may regulate genes that encode proteins involved in the resistance mechanisms described above by excretion of metals via efflux transport system, such as CpxR/CpxA that is similar to the RND-efflux pump GesABC.

CHAPTER III: METHODS

3.1 Bacterial Strains

Stenotrophomonas maltophilia (ATCC 53510) was obtained from AATC the American Type Culture Collection (Manassas, VA). The *Escherichia coli* EC100D™ *pir*-116 (EC6P095H) was acquired from Epicentre (Madison, WI), a subsidiary of Illumina.

4.2 Growth Medium and Gold Salts

Lennox LB medium was obtained from Growcells (Irvine, CA) containing 10 g tryptone, 5 g yeast extract and 5 g NaCl per liter. All plating methods were done with Amresco (Solon, OH) bacteriological agar at 16 g/L. When required, LB medium was supplemented with 50 µg/ml kanamycin (Amresco, Solon, OH) for *E. coli* and 800 µg/ml kanamycin for *S. maltophilia* 02. Gold salts, Au(III)Cl [Au_2Cl_6], were acquired from Amresco, Solon, OH.

3.3 Preparation of Electrocompetent Cells

2 ml of overnight culture (from drum-roller at 4 rpm) of *S. maltophilia* O2 strain was added to 100 ml of LB medium. The cells were grown at 30^0C in a shaker until they reached an optical density (OD) of between 0.6 - 1.0 at a wavelength of 600 nm, measured with Biophotometer (Eppendorf; Hauppauge, NY). The cells were then transferred to sterile 50 ml centrifuge tubes that were pre-chilled on ice. The cells were centrifuged at 7,000 x g for 10 minutes at 4^0C; supernatant was poured off. The cells

were resuspended in approximately 15 ml of chilled sterile water, mixed with more chilled sterile water up to 80 ml and centrifuged at 5000 x g at 4^0C for 15 minutes. This wash step was repeated twice and after the final wash, the cells were resuspended in 200 µl of chilled 10% glycerol and distributed into 1.5 ml microcentrifuge tubes.

3.4 Transformation using the $CaCl_2$ method

An overnight culture of *Escherichia coli* strain EC100D™ *pir*-116 was grown at 37°C with agitation at 120 rpm. 10 ml of the cells were added to 200 ml of fresh LB media. This was grown using the same temperature and agitation to an optical density ($OD_{600\ nm}$) of 1.0. The cells were then transferred into two 50 ml centrifuge tubes and placed on ice for 10 minutes. Once cooled, the cells were pelleted at 4°C and 5,000 X g for five minutes. Following centrifugation, supernatant was then discarded and the cells were then resuspended using 2 ml of ice-cold transformation buffer containing 15% glycerol (v/v), 0.1 M $CaCl_2$, 10 mM Tris-HCl (pH 8.0), and 10 mM $MgCl_2$. Once cells were resuspended, 400 µl of cells were transferred to individual 1.5 ml microcentrifuge tubes and incubated on ice in a refrigerator (4°C) overnight. After incubation, the generated calcium chloride competent cells were frozen at -80°C for future use.

3.5 Transposon Mutagenesis

In a sterile 1.7 ml microcentrifuge tube, 40 µl of electrocompetent cells were mixed with 0.5 µl of the EZ-Tn5™ Transposome. The control was supplemented with sterile water in

place of the transposome. The cell-transposome mixture was added to an electroporation cuvette with a 2 mm gap previously cooled at -20°C and tapped to the bottom. The cells were pulsed at 25 μF, 200 ohms, and 2.5 kV using a Bio-Rad™ Pulse Controller Plus. Immediately after the shock, 960 μl of SOC medium was mixed by pipetting up and down gently. The SOC medium consisted of 0.5% (w/v) yeast extract (AMRESCO; Solon, OH), 2% (w/v) tryptone (AMRESCO), 10 mM NaCl (AMRESCO), 2.5 mM KCl (AMRESCO), 10 mM $MgCl_2$, 20 mM $MgSO_4$ (Thermo Fisher Scientific; Waltham, MA), and 20 mM Glucose (AMRESCO). Electroporated cells were then transferred to a sterile 1.7 ml tube and incubated at 30°C for 45-60 minutes in a shaking incubator (120 rpm). After this incubation, 100 μl of the cell mixture was spread onto LB plates containing 800 μg/ml of kanamycin and incubated overnight at 30°C to select for the successful transformants. Colonies were picked with sterile toothpicks and gridded onto new LB plates made with 800 μg/ml of kanamycin and incubated overnight at 30°C. The gridded plates were replica plated onto plates containing 800 μg/ml of kanamycin (LB) and 50 μM gold chloride (R3A-tris medium). Gold sensitive mutants exhibited an ability to grow on the LB kanamycin plates but not on gold chloride plates.

3.6 Genomic DNA Preparations

Genomic DNA was purified through single colony previously grown overnight in 20 ml LB supplemented with 800 μg/ml kanamycin at 30°C with agitation (120 rpm). 10 ml of overnight culture was pelleted by centrifugation at 5,000 X g for 3 minutes at 25°C. After

the supernatant was poured off, cells were resuspended in 2 ml of TE buffer (10 mM tris-HCl (pH 8.0) and 1 mM EDTA). Addition of 5 µl of RNase A (AMRESCO; Solon, OH) and 5.5 ml of the genomic prep lysing solution (with addition of 50 mM tris-HCl and 3% SDS) with inversion completed the resuspension.

The genomic prep lysing solution was prepared using 9.8 ml of nuclease free water, 0.75 ml of 1 M tris-HCl (pH 8.0), and 4.5 ml of 10% SDS. After incubating mixture for 30 minutes at 65°C, 2 ml of warm 5 M NaCl were added to mixture. The mixture was then vortexed for 30 seconds and placed on ice for 15. The mixture was pelleted by centrifugation at 25°C at 10,000 x g. The supernatant was then poured into a sterile 50 ml tube and the DNA was precipitated using 6 ml of 100% isopropanol and mixed by inversion. A sterile glass rod was used to spool clumps of DNA precipitant to 500 µl of 70% (vol/vol) ethanol. DNA was pelleted via centrifugation at 13,000 X g for 1 minute at 25°C. The supernatant was poured off, and the DNA was resuspended in 500 µl of TE buffer and stored at 4°C for future use.

3.7 T4 DNA Ligation with Circular DNA Generation

Four separate reactions were set up with the following enzymes: *Sac* I. *Nco* I*, Eco*R I, and *Kpn I.* Each digestion reaction contained 2 µl 10X CutSmart buffer (New England BioLabs; Ipswich, MA), 5 µl of nuclease free water, 12 µl of the purified genomic DNA, and 1 µl of enzyme. The reactions were then incubated for 60 minutes at 37°C.

Generation of circular DNA via plasmid ligation from the DNA fragments was performed with the following methods: the digestion reactions were incubated in a thermocycler for 20 minutes at 80°C to inactivate the restriction endonucleases. The ligation reactions were carried out in 1.7 ml microcentrifuge tubes containing 10 µl of 10X T4 DNA ligase buffer, 73 µl of nuclease free water, 2 µl of T4 DNA ligase (New England BioLabs; Ipswich, MA), and 15 µl of digested DNA. After the DNA was added to the ligation mix it was incubated overnight at 4°C.

3.8 Plasmid Purification

A single transformant was picked and selected for overnight grown at 30°C in 5 ml LB supplemented with 50 µg/ml of kanamycin. The bacterial cells were then pelleted using a centrifuge at 8,000 X g and 4°C for six minutes. The supernatant was poured off and any excess liquid was pipetted out. Following this, the plasmid DNA was then purified using the Wizard Plus SV Minipreps DNA Purification System (Promega; Madison, WI), and then pellet was resuspended in Cell Resuspension Solution (250 µl). 250 µl of Cell Lysis Solution was added and mixed by inversion of the tube. The cells were incubated at room temperature for 5 minutes until the solution cleared, then 10 µl of Alkaline Protease Solution was added and mixed by inverting the tube four times. The cells were incubated again at room temperature for 5 minutes, and 350 µl of Neutralization Solution was added and mixed by inversion. The mixture was pelleted using a microcentrifuge at 14,000 rpm and 25°C for ten minutes. The cleared lysate was decanted into a spin column that was fitted into a collection tube. The two-part assembly was placed in a microcentrifuge at 14,000 rpm and 25°C for one minute, the spin column was removed, the flow-through was

discarded, and the spin column was placed back into the collection tube. 750 µl of Column Wash Solution was added to the spin column, and it was centrifuged at 14,000 rpm and 25°C for one minute. The spin column was removed, the flow-through was discarded, and the spin column was placed back into the collection tube. A final 250 µl of Column Wash Solution was added to the spin column, and it was centrifuged at 14,000 rpm and 25°C for one minute, with the spin column removed, the flow-through discarded, and the spin column was placed back into the collection tube like before. The two-part assembly was centrifuged at 14,000 rpm and 25°C for two minutes, and the spin column was transferred to a sterile 1.7 ml microcentrifuge tube. The plasmid was eluted from the spin column using 100 µl of nuclease free water and centrifuging at 14,000 rpm and 25°C for one minute. The spin column was discarded, and the purified plasmid was stored at -20°C.

3.9 Sequencing Prep

The concentration of DNA that would be used in each sequencing reaction was determined by NanoDrop Spectrophotometer (Thermo Fisher Scientific; Waltham, MA). First the NanoDrop was blanked with 2 µl of nuclease free water. Then, 2 µl of DNA was placed on the ocular pedestal for measurements. The nucleic acid concentration, the measurements at the wavelengths of 260 nm (A_{260}) and 280 nm (A_{280}), and the ratio of the measurements at the wavelengths (A_{260}/A_{280}) were recorded. The DNA volume needed for each sequencing reaction was calculated by the amount of ng required for 50 fmol divided by the plasmid concentration. The volume of ng required to obtain 50 fmol of plasmid in the sequencing reaction was determined by comparing the estimated size of the plasmid that

was found upon digesting the plasmid to a table provided by the sequencing kit. The DNA was then mixed with nuclease free water to raise the total volume of the reaction to 10 µl.

A 0.2 ml PCR tube (GeneMate; Lodi, CA) was added with 50 fmol of DNA and water in a final volume of 10 µl. Tubes containing the reaction were then incubated at 96°C in a thermocycler for 1 minute and then set to cool to room temperature. Next, 8 µl of the DTCS quick start mix was added to each tube. 2 µl of a 1.6 µM primer was then added to the corresponding tubes. The mixtures were then placed into a thermocycler and sequencing reaction was carried out with 30 total cycles at 96°C for 20 for DNA denaturation, 50°C for 20 seconds for primer annealing, and 60°C for 4 minutes for optimal elongation by DNA polymerase. The reactions were then held at 4°C for the remaining time. Sequence reactions were separated using a Beckman Coulter CEQ 2000XL DNA analysis system (Beckman Coulter; Fullerton, CA).

Primer	**Nucleotide Sequence**
KAN-2 FP-1	ACCTACAACAAAGCTCTCATCAACC
R6KAN-2 RP-1	CTACCCTGTGGAACACCTACATCT
A12 F1	CCTGACCAGCTACGAGTACAA
A12 R1	CCTTTGCAAATTCTTGGTTTGG
A12 F2	GTGCTGGAGTACCTGATGATG
A12_R2	GCGGTAGCGTAGTTCTTCAG

A12_F3	GCCTGAAGAACTACGCTACC
A12_R3	GGGATCGCGAAACGGTAG
A12_F6	CATGAACAACCTGGTCTCCTAC
A12_F5	CTGACCGACAGCATCAAC
A12_F8	AGGACCTGATCAAGGACTACC
A12_F7	AAAGGTCTATGCCTCCAAGG
A12_F9	GATCGTGCAGGACCTGAT
A12_F10	GACATCGGCAAGGTGCT

3.10 Sequence Analysis

GeneStudio Professional was used to convert the raw data from the sequencer into the FastA and plain text formats. The nucleotides of the transposon were removed by copying the sequences into Microsoft Word and searching for the nucleotides at the 3' end of the transposon: 5' – GAGACAG – 3'. All of the nucleotides beginning at the 3' G through the 5' end of the transposon insert were deleted. The remaining nucleotides belonged to the interrupted gene. Each nucleotide sequence was analyzed using the Basic Local Alignment Search Tool (BLAST) (39) [Figure 1], specifically a nucleotide BLAST (BLASTn) to determine a tentative identification of the gene that was interrupted. A blastn search compared the query sequence to other sequences in the National Center for Biotechnology Information (NCBI) database to find similar sequences that are ranked by how identical the query is to the found sequences. Next, the contig program in GeneStudio Professional

was used to align multiple overlapping sequences to obtain a consensus sequence. The consensus sequence was analyzed using BLASTn and BLASTx to translate and identify coding sequences. The coding nucleotide sequence with the highest similarity to the consensus sequence was found and downloaded. The downloaded sequence was entered into GeneStudio Professional to be used as a reference in correcting ambiguities and forming a better consensus sequence. The consensus sequence was uploaded to Genome Compiler for further analysis.

A multiple sequence alignment was generated to determine the homology of the protein coding region of the identified gene. First, the coding DNA sequence of the mutant was translated in Genome Compiler to the amino acid sequence. Then, the amino acid sequence was analyzed using BLASTp.

3.11 Minimal Inhibitory Concentrations (MICs)

The Minimal Inhibitory Concentration (MIC) of selenite for the mutant (A12) and the wildtype (*S. maltophilia* 02) were determined by liquid culture experiments. Overnight cultures of were prepared by picking a single colony from a streak plate and placing it into 5 ml of LB. The mutant culture was supplemented with 80 µl of kanamycin. The cultures were grown at 30°C for 18 hours in a roller drum (Fisher Scientific; Waltham, MA). Following incubation, cultures were diluted into fresh LB broth in a 1:50 ratio.

Gold-chloride (AMRESCO, ACS Grade; Solon, OH) was added to each tube in the following concentrations: 0 µM M, 10 µM, 50 µM, 100 µM, 150 µM, and 200 µM. A Klett

Colorimeter (Fisher Scientific; Waltham, MA) was used to measure the turbidity of the cells. Then, the cultures were incubated at 30°C for 18 hours in a roller drum. Turbidity was measured again following the incubation and the minimal inhibitory concentration of gold chloride was determined for the A12 mutant and the wild type strain. This step was repeated several times with varying gold chloride concentrations in order to determine accurate concentrations for MIC determination. The MICs were calculated by first taking the average of the turbidity measurements at each concentration of sodium chloride. In Microsoft excel, the average measurements were used to calculate the standard deviation and the standard error.

3.12 Agarose Gel Electrophoresis

A 1% agarose gel was prepared by adding 1.3 g of BioExcell Agarose LE (WorldWide Medical Products; Bristol, PA) to 130 ml of 1X TBE (AMRESCO; Solon, OH). The 1X TBE buffer was made by diluting 10X TBE buffer (0.089 M Tris Base, 0.089 M Borate, and 0.002 M EDTA) with water. The mixture was subsequently heated in a microwave until the agarose has uniformly dissolved, then 13 µl of GelGreen Nucleic Acid Stain (Embi Tec; San Diego, CA) was added and mixed using a stir bar and stir plate. Once mixed, the contents were poured into a gel tray and combs were inserted to form wells and removed after the gel hardened. The gel was transferred to a RunOne Electrophoresis System and covered with 1X TBE buffer. All gels preformed using this method were ran at 100V for 30 minutes.

CHAPTER IV: Results

4.1 Generation of the A12 mutant

Introduction of the EZ-Tn5 transposome by electroporation into the genome of *S. maltophilia* 02 resulted in a gold-sensitive mutant A12 (Figure 1). This transposon randomly incorporated itself into *S. maltophilia* 02, producing several thousand kanamycin resistant transformants. Each transformant contained a minimum of 1 transposon insert, requiring growth on medium supplemented with 800 μg/ml kanamycin. *S. maltophilia* 02 was already resistant to 50 μg/ml kanamycin. Increasing the kanamycin concentration to 800 μg/ml kanamycin allowed for the selection of transposon inserts. Replica plating of 880 transformants onto 10 LB plates containing 800 μg/ml kanamycin and R3A tris plates containing 30 μM gold chloride resulted in a single sensitive *S. maltophilia* 02 mutant, A12.

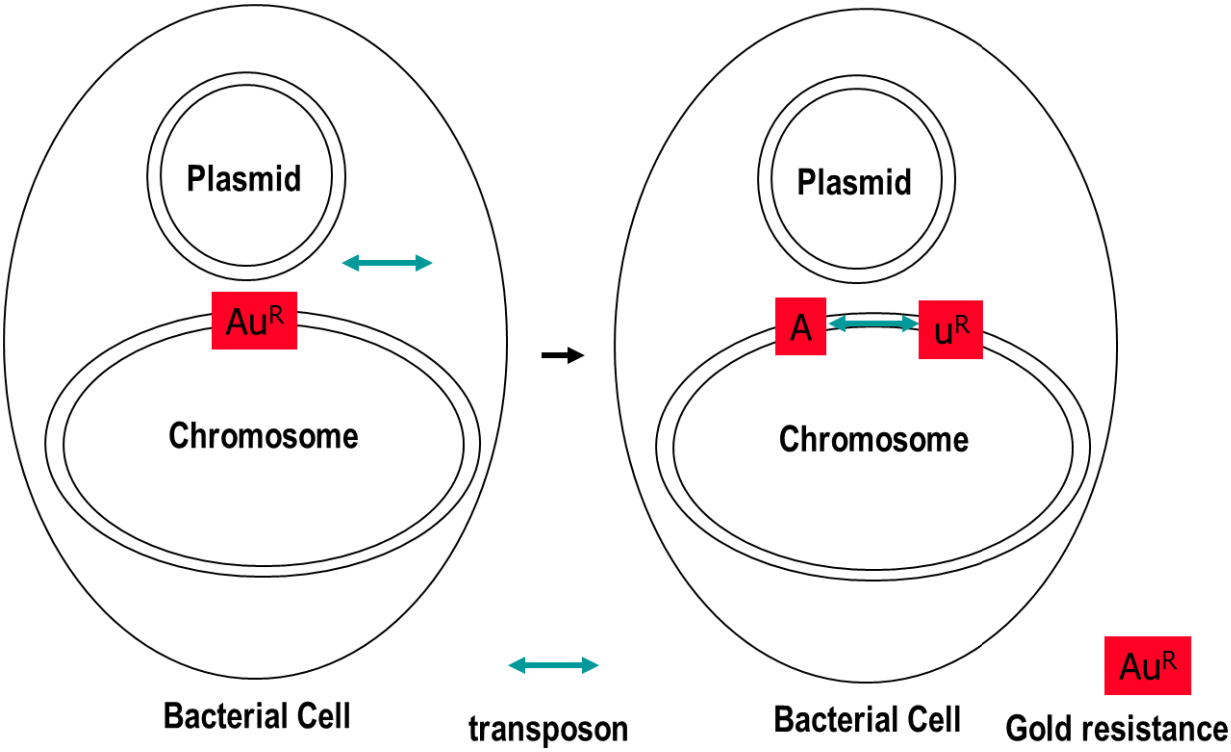


Figure 1- The transposon inserted into the S02 genome, interrupting a gene involved in gold resistance

4.2 Minimum Inhibitory Concentrations (MIC's):

The next step was to measure the mutant's level of gold sensitivity using MIC experiments. Overnight cultures (18 hours) of **A)** *S. maltophilia* 02 and **B)** A12 were diluted into fresh R3A-tris medium (1:50). Different concentrations of gold chloride were added, and the cultures were incubated an additional night. Turbidity (Klett units) was plotted against the gold concentrations. The MIC for the A12 mutant was 175 µM,

whereas the wild type grew well at 190 µM gold. This showed that A12 is still somewhat resistant to gold chloride but not as resistant as *S. maltophilia* 02 (Figures 1-2).

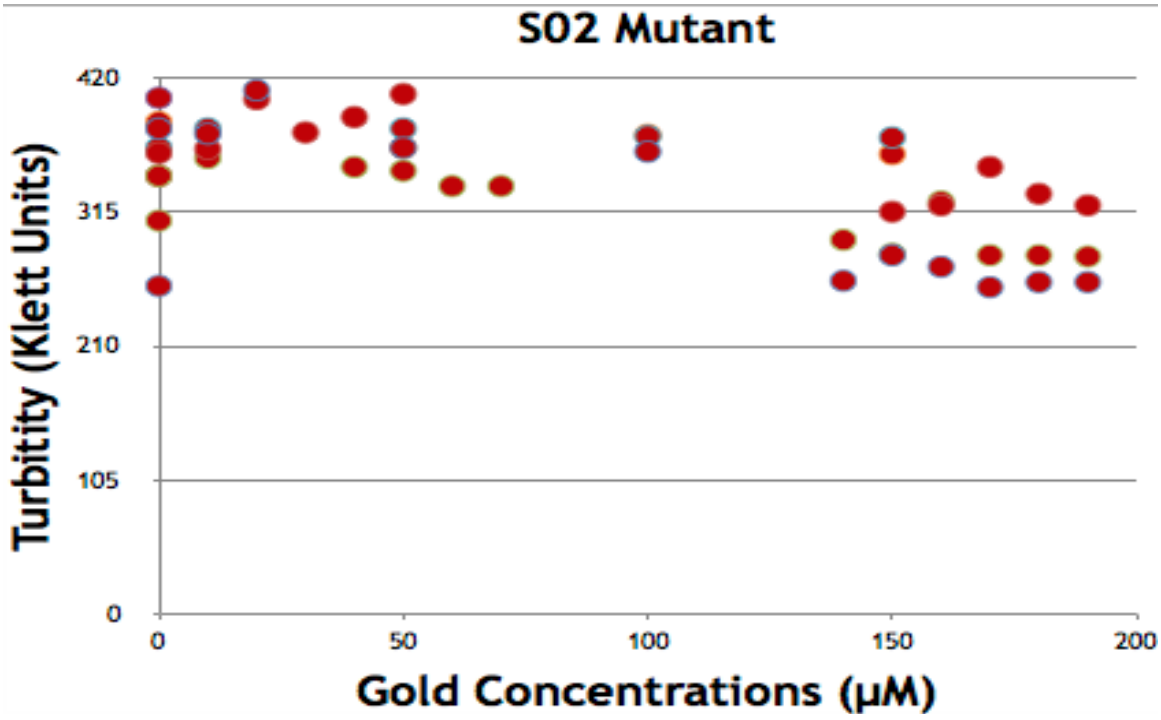


Figure 2: Minimum Inhibitory concentration of *Stenotrophomonas maltophilia* (S02) wild-type

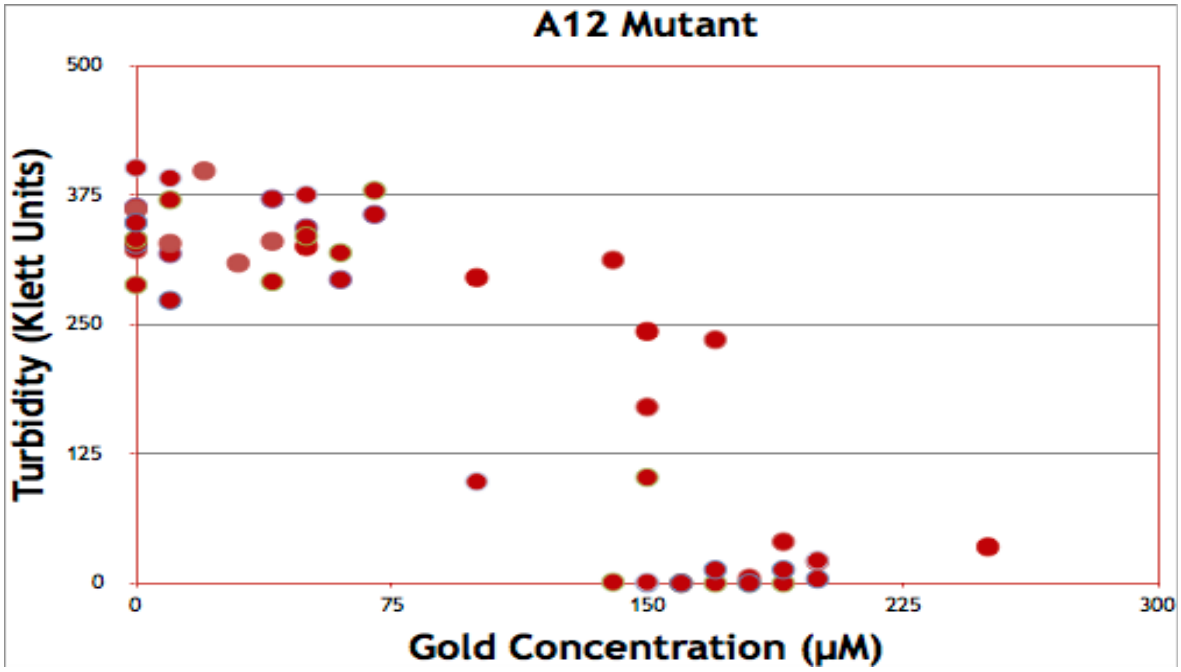


Figure 3: *Minimum Inhibitory concentration of Stenotrophomonas maltophilia (A12) mutant*

4.3 Sequencing A12 Mutant Gene

Gene rescue was performed to tentatively identify the interrupted gene sequence of our mutant. Genomic DNA was purified from the A12 mutant and digested with the restriction endonucleases *Sac* I, *Kpn* I, *Nco* I, *Eco*R I, and *Aat* II, which did not cut inside the transposon. The digested DNA appeared as a smear, ranging from larger than 10 kb to smaller than 500 bp in size on an agarose gel (Figure 4A-B). To isolate the fragments containing the inserted transposon flanked by a region of the interrupted chromosome, the digested DNA was ligated using T4 DNA ligase, transformed into *E. coli* strain ECD100D *pir116* and plated on LB plates containing 50 μg/ml kanamycin. Plasmids

containing the R6Kγ replication origin require the *pir* gene to replicate in *E. coli*. The *pir116* gene is a *pir* mutant that allow for high copy number. The only transformants that will grow are the ones that contain a recombinant plasmid with the transposon and flanking chromosomal region.

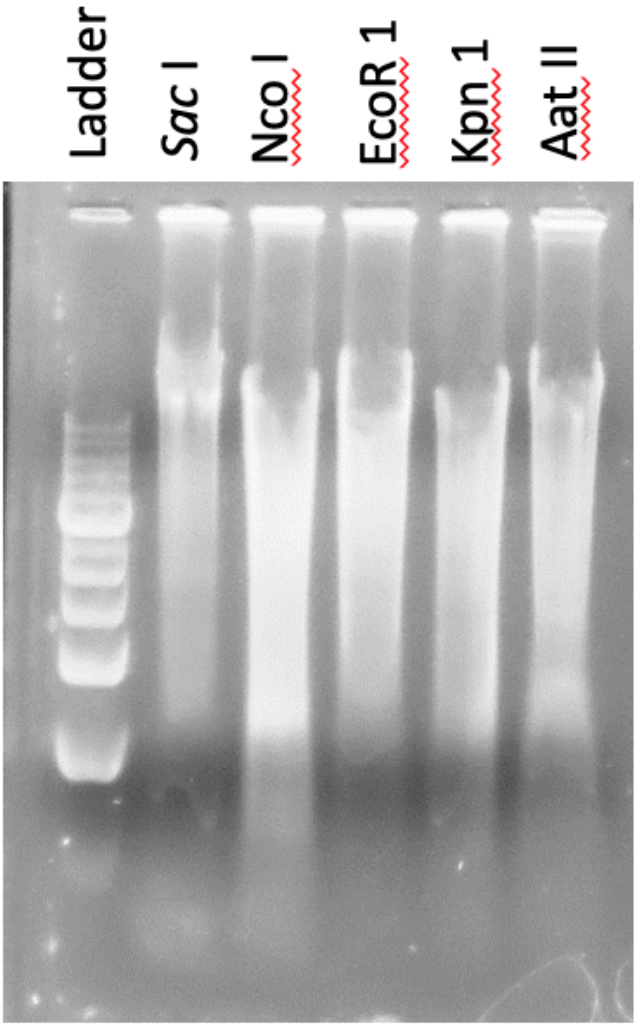


*Figure **4A:** Genomic DNA purified from A12 was digested with enzymes which cut outside the transposon. In order, Lane 1. 1Kb ladder, lane 2. Sac I, lane 3. Nco I, lane 4. EcoR I, lane 5. Kpn I, lane 6. Aat II.*

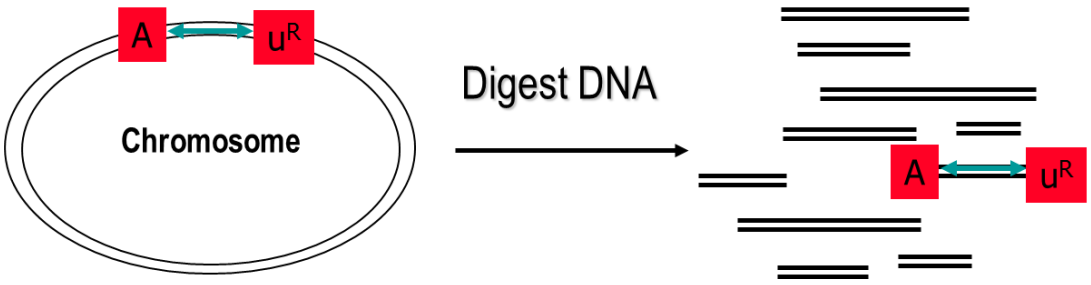
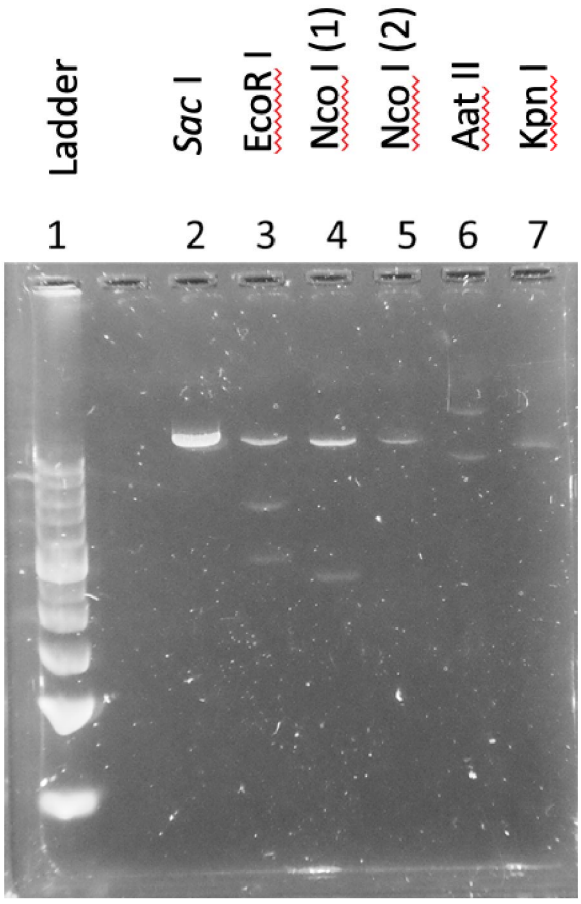


Figure 4B- The transposon inserted into the S02 genome, interrupting a gene involved in gold resistance. Genomic DNA is purified from the gold-sensitive mutant and is digested with enzymes that do not cut the transposon

The recombinant plasmids were purified from transformants and digested by the same enzyme they were formed from the genomic DNA digestion. This was used to estimate the size of the plasmids to calculate the volume of DNA required for sequencing reactions. Agarose gel electrophoresis was used to estimate the size of each plasmid from the following: *Sac1, NcoI, Ecor1, Kpn1,* and *Aatt II* (Figure 5). The estimated size of each plasmid was above 10 kb.



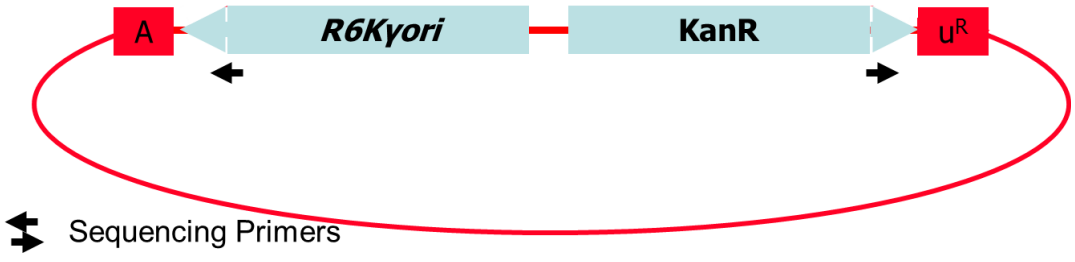


Figure 5:. *Genomic DNA was ligated with T4 DNA ligase and transformed into competent E. coli to generate recombinant plasmids containing a gene interrupted by the transposon. In order, Lane 1. 1Kb ladder, lane 2. Sac I, lane 3. Nco I, lane 4. EcoR I, lane 5. Kpn I, lane 6. Aat II, lane 7. Kpn I*

The generated plasmids were then sequenced using primers homologous to the transposon (Table 1). Additional primers were designed to sequence additional regions of the interrupted gene (Table 1), and a 2,713 bp contig of the region was assembled use Contig Editor in GeneStudio (Appendix A). BLAST analysis of the resulting sequence showed that it was homologous to a DNA segment from *S. maltophilia* strain OUC_EST10 (OUC_EST10) (Figure 6).

4.4 Sequence Alignment

The OUC_Est10 sequence was imported into Genome Compiler and used as a reference sequence to map the A12 mutated region. Figure 6 shows an alignment of the A12 sequence (bottom line) with the reference sequence (top line). The green bars in the A12 line shows that the *S. maltophilia* 02 sequence is identical to genes for a DNA binding protein and a two-component sensor histidine kinase. The red bar indicates differences in a hypothetical protein. The light blue bar indicates the transposon insertion site seven base pairs after the stop codon of the hypothetical protein and 65 base pairs before the start codon of the gene for the DNA binding protein. It also inserted a 10 bp repeat, GAACAGGGAT, next to the transposon and 65 base pairs before the start codon of the DNA binding protein. Thus, this possibly interferes with the expression of the DNA binding protein and the two-component sensor kinase. BLAST analysis did not reveal a possible function for the hypothetical protein. The nucleotide sequence of the mutagenized A12 region is in the appendix.

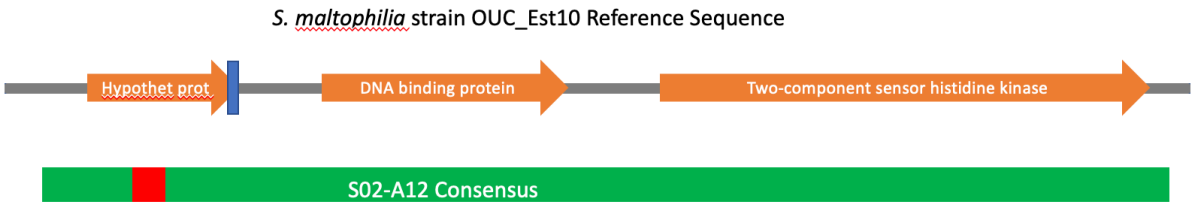


Figure 6: A12 Consensus Sequence aligned with the reference sequence. The transposon inserted itself seven base pairs after the stop codon of a hypothetical protein (blue box) and 65 base pairs downstream of genes for a DNA binding protein and a two-component sensor histidine kinase. The red box indicates a region of mismatch with the reference strain.

4.5 BLAST Analysis

To determine if the *S. maltophilia* 02 hypothetical protein might be related to other known proteins, its amino acid residue sequence was analyzed by BLAST (Fig 7). It was 100% identical to other *S. maltophilia* hypothetical proteins, but none of the results suggested a function for this protein.

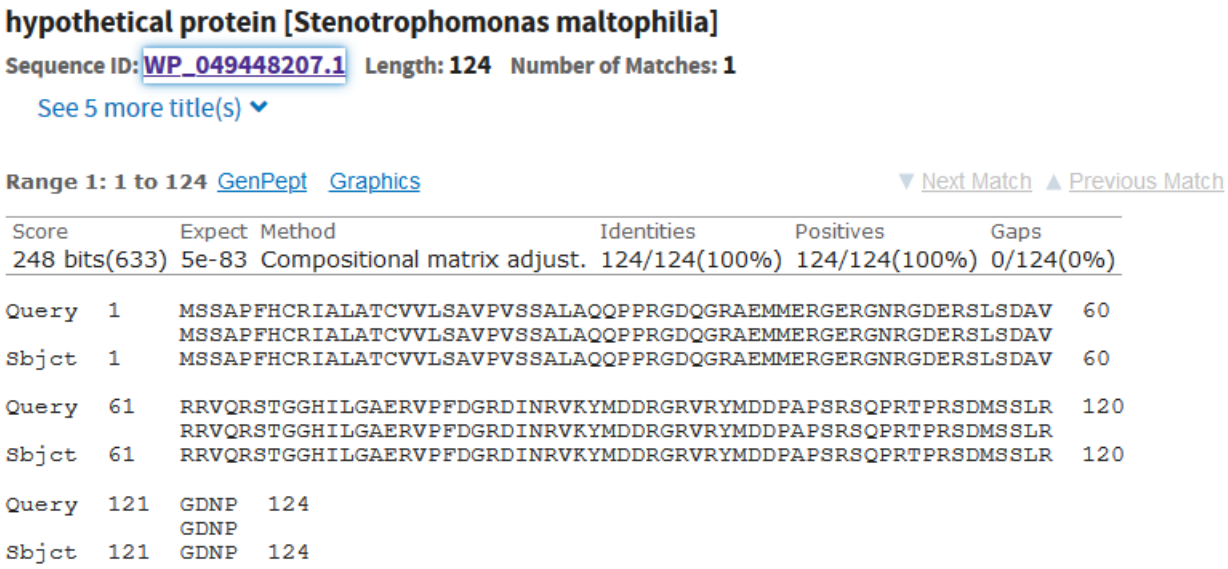


Figure 7. BLAST analysis of the hypothetical protein from *S. maltophilia* 02. The transposon inserted seven base pairs after the stop codon of a hypothetical protein.

Perhaps the transposon insertion prevented the expression of the downstream DNA binding protein or two-component sensor histidine kinase. As previously mentioned, gold resistance in *Salmonella*, is partially regulated by the CpxR/CpxA two component sensor (5). Figure 8 shows a BLAST comparison between the *S. maltophilia* 02 DNA binding protein with CpxR from *Salmonella.* Although they are only 58% similar, the results suggest that the *S. maltophilia* 02 DNA binding protein could have a similar function to CpxR from *Salamonella.* BLAST analysis of the *S. maltophilia* 02 DNA binding protein showed that it is identical to a *S. maltophilia* OmpR, a regulatory protein (15, 21).

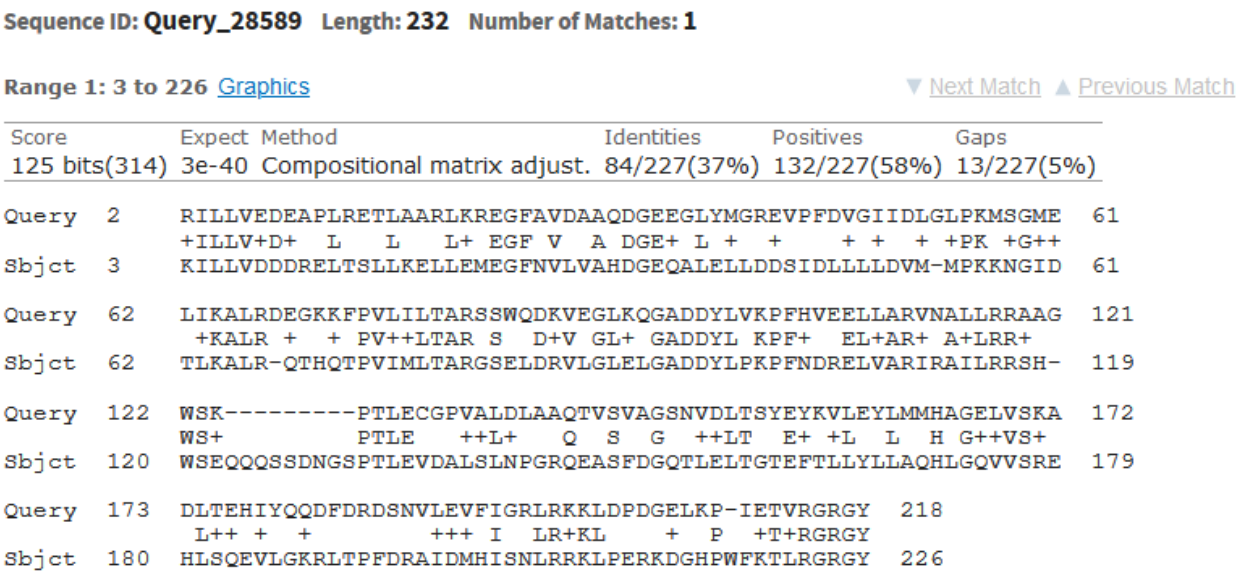


Figure 8. BLAST alignment the putative, *S. maltophilia* 02 sensor protein with the CpxR protein from *Salmonella*.

Figure 9 shows a BLAST comparison between the *S. maltophilia* 02 histidine kinase with CpxA from *Salmonella.* Although they are only 47% similar, the results suggest that the *S. maltophilia* 02 histidine kinase could have a similar function to CpxA from *Salamonella.* BLAST analysis of the *S. maltophilia* 02 histidine kinase showed that it is 99% similar to a *S. maltophilia* PhoQ sensor which is involved in antibiotic resistances (40).

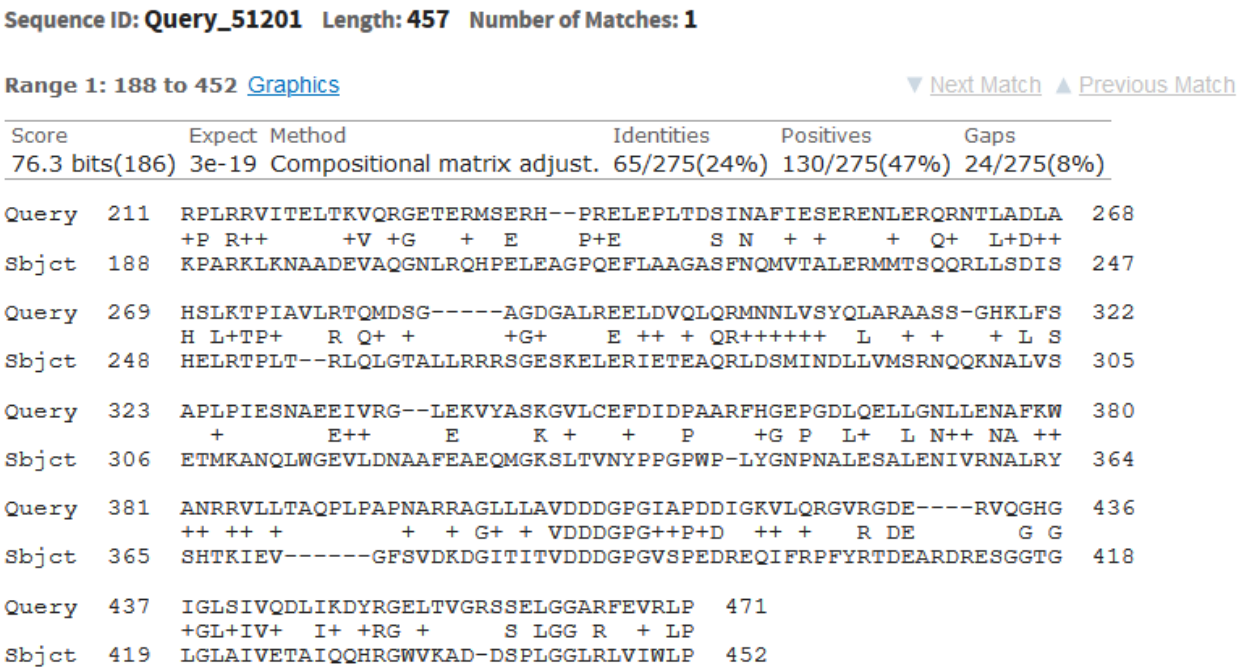


Figure 9. BLAST alignment the putative, *S. maltophilia* 02 sensor protein with the CpxA protein from *Salmonella*.

CHAPTER V: DISCUSSION

I postulated that the transposon inserted itself into a region of DNA that may regulate genes that encode proteins involved in the resistance mechanisms described above by excretion of metals via efflux transport system that is similar to the RND-efflux pump GesABC. After performing transposon mutagenesis, we observed a mutant colony unable to grow on gold-chloride supplemented medium. Liquid culture experiments showed that the minimal inhibitory concentration (MIC) for A12 was 170 µM gold chloride, compared to above 190 µM for the wild type. DNA sequencing and Basic Local

Alignment Search Tool (BLAST) analysis showed that the transposon inserted itself downstream of genes for a DNA binding protein and a two-component sensor histidine kinase. BLAST analysis revealed a sequence similar to the sensor protein PhoQ, one components of a two-component histidine kinase. PhoQ is a sensor protein involved in the control of acid resistance genes, virulence, and diverse cellular activities. Our data supports that transposon mutagenesis was able to interrupt a gene important in issuing resistance to high concentrations of gold.

5.1 CpxR/CpxA system of *Salmonella*

Transposon mutagenesis resulted in disruption of a region of DNA downstream of a DNA binding site and two-component sensor histidine-kinase. This region of DNA is in some way involved with conferring resistance to gold. The function of the hypothetical protein is not currently understood, but it is suspected to contribute to gold resistance within *S. maltophilia* 02. Few resistance mechanisms relating to gold have been researched at this time. One known is the GolS-operator within *Salmonella*, which uses a gold sensor histidine kinase CpxR/CpxA, a main stress-responding system for the cell-envelope. The CpxR/CpxA two-component system modulates the transcription in excess of fifty genes coding for chaperones, proteases, and envelope-associated complexes including multiple RND efflux pumps, many which are associated with metal homeostatic regulation.

Au(I) triggers activation of the CpxR/CpxA system and deletion of the *cpxRA* operon, which significantly reduces survival in with the toxic metal present. Interestingly, similar

to how the possible mutation of PhoQ in *S. maltophilia* 02 reduces survivability in high concentrations of metals, deletion of either cpxR or *cpx*A, or mutation of the CpxR-binding site located upstream of the GolS-operator in the *gesABC* promoter region reduces but does not fully eliminate the gold resistance (5). It is unknown if these genes are similarly present in *S. maltophilia*, although several potential homologs can be found within the *S. maltophilia* 02 genome with BLAST comparison against the gesABC associated genes. These homologues all have similarities under 60%.

Since the transposon inserted itself after the gene for the hypothetical protein downstream of the DNA-binding site and two-component sensor histidine kinase, we cannot be certain that the gene for the *S. maltophilia* 02 sensor kinase was mutated. It is likely that both *Salmonella* and *S. maltophilia* have other sensor proteins responsible to respond to the presence of toxic levels of gold which have not been researched at this time.

5.2 Histidine Kinase (Discussion and Future Work)

Blast analysis revealed a 474/474 100% positive match identity to a PhoQ sensor protein, which is consistent with the lowered heavy-metal resistance of the A12 mutant strain. PhoQ is responsible for and the control of acid resistance genes and has several binding sites for divalent cations such as magnesium, silver, and copper. Normally, gold exists as univalent cation. Since the transposon inserted itself after the stop codon of a hypothetical protein located downstream of genes for a DNA binding protein, the function of the

hypothetical is probably not impaired if it is involved in *S. maltophilia* 02 gold-resistance.

Reverse transcriptase-PCR (RT-PCR) may reveal if transposon insertion interfered with the expression of the hypothetical protein, *cpxR* or *cpxA* (*phoQ*). This experiment involves growing the wild type and A12 mutant to mid-log phase, then exposing them for 30 minutes to a solution of gold salts or a sterile water control, and purifying RNA from them. Then, RT-PCR reactions using primers for each gene and agarose gel electrophoresis will reveal the absence or presence of expression for these gene in the presence of gold. The presence of a PCR product in the wild type and the absence of a PCR product in the A12 mutant would suggest that transposon insertion interfered with gene expression.

Protein analysis may also reveal if the transposon interfered with protein expression in the mutated region. In this case, the same growth curve experiment on the wild type and the A12 mutant can be performed in the presence or absence of gold salts. Instead of purifying RNA after exposure to gold, protein from each sample is separated by one dimensional (1D) polyacrylamide gel electrophoresis (SDS-PAGE). The CpxR and CpxA (PhoQ) proteins are probably not expressed at high enough levels to detect protein bands but maybe the hypothetical protein and other gold resistance proteins can be detected by SDS-PAGE. The presence of a protein band in the separated gold-treated wild type sample and a lack of the corresponding band in the separated gold-treated A12 mutant sample would indicate that the transposon interfered with the expression of the observed

protein. The protein band would then be excised from the gel and identified by tandem mass spectrometry. These experiments were attempted without success (Appendix A). There were no consistent differences between expression profiles for each sample. They may require two-dimensional gel electrophoresis.

Sometimes, a transposon will incorporate into the genome in one or more places of the host genome. Southern blotting can determine if the transposon only inserted in one distinct site within the genome, and not multiple locations which could be affecting additional genes. In this experiment, genomic DNA from the wild type and the mutant is digested with enzymes that cut outside the transposon, with the bands separated by agarose gel electrophoresis and transferred to a nylon membrane. Then, a probe consisting of a part of the kanamycin gene from the transposon is labeled and hybridized to the membrane. If no bands appear in the lanes for the wild type and one band appears in the lanes for the A12 mutant, then the transposon inserted in only one place. The appearance of two or more bands for the mutant indicates more than one transposon insertion. This could also reveal that neither protein is responsible for the sensitive phenotype. Because of the 2020 COVID-19 pandemic, this experiment was started but not completed.

This research established generation of a gold-sensitive mutant strain of *Stenotrophomonas maltophilia* 02 to an otherwise heavy-metal resistant organism. Minimum inhibitory concentrations for sodium chloride were experimentally determined and can be used as a benchmark for future research. Transposon incorporation seven base

pairs after a hypothetical protein downstream of a two-component sensor histidine kinase PhoQ suggest there may be a negative impact on the ability for the efflux system to function properly. Research carried on the mutant and wild-type should first establish confirmation by Southern-blot that the transposon only had a single incorporation into the genome.

Chapter VI: Appendices

>lcl|Query_1305 unnamed protein product
CpxA-like S02
MSGRLWFFRRWRPRSLQARQMFAASVGLVAFLALAGYALDAAFADTAKAILRERLKNYATAYA
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EGPLPMIQIDGS
QGSVYRYGLGLVWDADADPATEFPYTIYVMEDSRALGAQLRVFRSRVWFYLGGIGLILLLLQTVI
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RT
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DDIGK
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CpxA - Salmonella
>WP_020845738.1 MULTISPECIES: envelope stress sensor histidine kinase CpxA [Salmonella]
MIGSLTARIFAIFWLTLALVLMLVLMLPKLDSRQMTELLDSEQRQGLMIEQHVEAELANDPPNDL
MWWRR
LFRAIDKWAPPGQRLLLVTSEGRVIGAERSEMQIIRNFIGQADNADHPQKKKYGRVEMVGPFSVR
DGEDN

YQLYLIRPASSSQSDFINLLFDRPLLLLIVTMLVSSPLLLWLAWSLAKPARKLKNAADEVAQGNLR
QHPE
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IETE
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YGNPNALESALENIVRNALRYSHTKIEVGFSVDKDGITITVDDDGPGVSPEDREQIFRPFYRTDEAR
DRESGGTGLG
LAIVETAIQQHRGWVKADDSPLGGLRLVIWLPLYKRS

CpxR-like S02

MRILLVEDEAPLRETLAARLKREGFAVDAAQDGEEGLYMGREVPFDVGIIDLGLPKMSGMELIKA
LRDEGKKFPVLILTARSSWQDKVEGLKQGADDYLVKPFHVEELLARVNALLRRAAGWSKPTLEC
GPVALDLAAQTVSVAGSNVDLTSYEYKVLEYLMMHAGELVSKADLTEHIYQQDFDRDSNVLEVF
IGRLRKKLDPDGELKPIETVRGRGYRFAIPRNEG

CpxR – Salmonella

>AYU74901.1 CpxR [Salmonella enterica subsp. enterica serovar Enteritidis]
MNKILLVDDDRELTSLLKELLEMEGFNVLVAHDGEQALELLDDSIDLLLLDVMMPKKNGIDTLKA
LRQTH
QTPVIMLTARGSELDRVLGLELGADDYLPKPFNDRELVARIRAILRRSHWSEQQQSSDNGSPTLEV
DALSLNPGRQEASFDGQTLELTGTEFTLLYLLAQHLGQVVSREHLSQEVLGKRLTPFDRAIDMHIS
NLRRKLPERKDGHPWFKTLRGRGYLMVSAS

Appendix A: Contig Assembly DNA Sequence

GGATTCACTGCCCCAAACCAAGAATTTGCAAAGGATTTGTAAAACACCGGGC

CCCGAACCCGGCACCCGGATTTACGACTTTTGAACGAATCGCCGTGCTCGGC

TAGGATCGCATCGATGTCTTCCGCTCCCTTCCATTGCCGCATTGCCCTGGCCA

CCTGCGTGGTGCTGTCGGCTGTGCCGGTGTCATCGGCGCTGGCGCAACAGCC

GCCGCGTGGCGATCAGGGGCGTGCAGAGATGATGGAGCGGGGCGAGCGCGG

CAACCGCGGCGACGAACGTTCGCTGTCCGATGCCGTGCGCCGCGTGCAGCGC

AGCACCGGTGGCCACATCCTCGGCGCCGAGCGCGTTCCGTTCGACGGTCGTG

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AGCCGACGCTGGAGTGCGGTCCGGTTGCCCTGGACCTGGCTGCGCAGACTGT

CAGCGTCGCCGGCAGCAATGTCGACCTGACCAGCTACGAGTACAAGGTGCTG

GAGTACCTGATGATGCACGCCGGTGAACTGGTCTCCAAGGCCGACCTCACCG

AGCACATCTACCAGCAGGACTTCGACCGCGACTCGAACGTGCTGGAGGTCTT

CATCGGCCGCCTGCGCAAGAAGCTGGACCCGGATGGCGAGCTGAAGCCGATC

GAGACCGTGCGCGGCCGCGGCTACCGTTTCGCGATCCCGCGCAACGAGGGCT

GAGCCGGCTCACCCTGGCGATTACACGATGTCCGGCCGTCTGTGGTTCTTCCG

ACGCTGGCGGCCGCGCTCACTGCAGGCGCGCCAGATGTTCGCCGCGTCCGTG

GGCCTGGTCGCGTTCCTGGCGCTGGCCGGTTACGCACTCGACGCCGCCTTCGC

CGATACGGCGAAGGCGATCCTGCGTGAGCGCCTGAAGAACTACGCTACCGCC

TACGCGGCCGGCATCGACTTCACCCGCGACCGCTCGCTGTACATCCGCGAGC

AGCCGCCGGATTCGCGCTTCGACGTGCCGGGCAGTGGCCTGTACCTGCAGGT

AGTGATGCCGCACGGCAAGGGCAATTCGATGTCCGCCGAAGGCCCGATGCTG

CCCACCGTCGGCGGCGGCCTGCTGGCACCGCGCCAGGAAGTGTTCGAAGGTC

CGCTGCCGATGATCCAGATCGATGGCAGCCAGGGCTCGGTGTATCGCTATGG

CCTGGGCCTGGTGTGGGATGCCGACGCCGATCCCGCCACCGAATTCCCGTAC

ACCATCTATGTGATGGAAGACTCGCGCGCGCTGGGTGCGCAGCTGCGGGTGT

TCCGCAGCCGGGTCTGGTTCTACCTGGGTGGCATCGGCCTGATCCTGCTGCTG

CTGCAGACCGTCATCCTGCAGTGGAGCCTGCGGCCGCTGCGTCGCGTGATCA

CCGAGCTGACCAAGGTGCAGCGCGGCGAAACCGAGCGCATGAGCGAGCGCC

ACCCGCGCGAGCTGGAGCCGCTGACCGACAGCATCAACGCCTTCATTGAAAG

CGAGCGCGAGAACCTCGAGCGCCAGCGCAATACCCTGGCCGACCTGGCGCAC

AGCCTGAAGACGCCCATCGCCGTGCTGCGCACGCAGATGGACAGCGGTGCCG

GCGATGGCGCCCTGCGCGAGGAGCTGGACGTGCAGCTGCAGCGCATGAACA

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CGATGGCCCGGGCATTGCCCCGGACGACATCGGCAAGGTGCTGCAGCGTGGC
GTGCGTGGTGACGAGCGCGTGCAGGGCCACGGCATCGGCCTGTCGATCGTGC
AGGACCTGATCAAGGACTACCGCGGCGAACTGACCGTCGGCCGTTCCAGCGA
ACTGGGCGGCGCCCGCTTCGAAGTGCGTTTGCCGCCGGGGCCGTAGCCTTGT
GTAGAGTC

> Hypothetical Protein sequence

MSSAPFHCRIALATCVVLSAVPVSSALAQQPPRGDQGRAEMMERGERGNR
GDERSLSDAVRRVQRSTGGHILGAERVPFDGRDINRVKYMDDRGRVRYMD
DPAPSRSQPRTPRSDMSSLRGDNP

*Appendix B: Future Growth Curves

The following method was intended for use in determining what proteins were being expressed when *S. Maltophilia* would be hitting a theoretical logarithmic-phase (exponential phase) growth. It was not included in the final paper due to lab foreclosure due to COVID-19 (2020 coronavirus) and subsequent shutting down of labs and associated research functioning.

Two different growth curves were used to measure the amount of proteins expressed during different times in an attempt to discern log-phase growth for the organisms. Then, gold was added right at the log-phase during rapid cellular division. Protein gels would be run to measure how much protein was produced, as well as the molecular weight of

the proteins to determine what proteins were being expressed. This could be used to determine which genes are turning on and expressing proteins, by visualizing what proteins are being expressed during cell death.

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