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**Metal-Citrate Transport in the Gram-Positive
Bacterium *Kineococcus radiotolerans***

A Capstone Project Submitted in Partial Fulfillment of the
Requirements of the Renée Crown University Honors Program at
Syracuse University

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and Renée Crown University Honors
May 2013

Honors Capstone Project in Biology

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Abstract

Little is known about metal-citrate transport in Gram-positive bacteria. Previously, all such transporters identified have belonged to the citrate-metal hydrogen symport (CitMHS) family. Presented herein is our research on the predicted metal-citrate transport system in the Gram-positive bacteria *Kineococcus radiotolerans*. Given the high alkaline natural environment of this organism, it was hypothesized such a metal-citrate transporter may utilize an antiport mechanism, which to date has not been observed. Herein it is shown that *K. radiotolerans* has a calcium-citrate transporter. Preliminary results also support the hypothesis that this transporter operates through hydroxide antiport, rather than hydrogen symport, making this the first example of such transport for CitMHS reported to date. In future work we hope to confirm this and knock out the purported gene for this protein through use of a modified TargeTron system.

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Chapter 1

Introduction

Citrate is an integral component of the TCA cycle and a significant carbon energy source. Because of this, many organisms have adapted transport mechanisms to acquire citrate in its different forms. Many bacteria utilize citrate transporters to take up free citrate - citrate coupled to sodium or protons. These proteins, however, cannot take up citrate complexed to di- and trivalent metal ions. Some species of bacteria have evolved a transporter that can recognize these complexes, and specifically transports divalent metal ion-citrate complexes.¹ In the proteins thus far identified, the metal-citrate complex is symported with hydrogen ions. The proteins have therefore been classified as CitMHS, or Citrate Metal Hydrogen Symport, proteins. To date, several of these proteins have been identified in various Gram-positive organisms, as shown in Table 1.

Bacteria	Protein	Metals Transported
<i>Bacillus subtilis</i> ²	Cit _{Bs1}	Mg ²⁺ , Ni ²⁺ , Mn ²⁺ , Co ²⁺ , Zn ²⁺
<i>Bacillus subtilis</i> ³	Cit _{Bs2}	Ca ²⁺ , Ba ²⁺ , Sr ²⁺
<i>Streptococcus mutans</i> ⁴	Cit _{Sm}	Fe ³⁺ , Mn ²⁺
<i>Enterococcus faecalis</i> ⁵	Cit _{Ef}	Ca ²⁺ , Sr ²⁺ , Mn ²⁺ , Cd ²⁺ , Pb ²⁺
<i>Streptomyces coelicolor</i> ⁶	Cit _{Sc}	Fe ³⁺ , Ca ²⁺ , Pb ²⁺ , Ba ²⁺ , Mn ²⁺

Table 1. Gram-positive bacteria identified as having CitMHS members, as well as the metal ions transported when complexed to citrate. Although CitMHS members have been postulate to exist in Gram-negative bacteria, at present none have been identified.

One putative CitMHS member has been identified in the bacterium *Kineococcus radiotolerans*, first discovered in radioactive uranium waste facilities.⁷ This organism is highly tolerant to radiation, as it has devised a mechanism to repair itself after sustaining damage to its DNA.⁷ It is believed that this protein could be used in the bioremediation of toxic metals; if the transporter was found to take up radioactive metal-citrate complexes, or could be modified to do so, it could potentially take up these complexes and remain unharmed due to its high tolerance for radiation.

To better understand these organisms and the CitMHS protein family, further research and knowledge is essential. Methods developed for use with the harmless *Kineococcus radiotolerans* could also be modified for use with more hazardous potential CitMHS members, including *Bacillus anthracis* and *Staphylococcus aureus*. Knowledge of the CitMHS members in these organisms could lead to development of new detection methods or vaccines. Further knowledge of these proteins could be integral to the future development of new medical and scientific advances.

Chapter 2

Materials and Methods

Materials

Broth and buffer materials were purchased from Becton Dickinson and Company, VWR, Sigma-Aldrich, or Merck. Materials were of biological grade and prepared with distilled and deionized water. Chelex 100 (15 g/L) was added to solutions to complex to any metals in solution and allowed to stir for a minimum of 24 hours. Chelex was removed by vacuum filtration, leaving the solution devoid of metals. Metal salts were of at least 99% purity and were purchased from Sigma-Aldrich. DNA was isolated using the Promega Wizard Plus SV Miniprep kit or the Omega EZNA Plasmid Mini kit. Primers were ordered from Integrated DNA Technologies. DNA was sequenced by the DNA Sequencing Core Facility or by Genewiz at Upstate Medical University in Syracuse, New York. 2 log DNA ladder #N0469S was purchased from New England Biolabs. Protein markers (broad range, 2-212 kDa) were ordered from New England Biolabs. Kaleidoscope protein markers were purchased from Bio-Rad. All cells were incubated in a Max^o 5000 or 4000. Cell density was determined using a Cary 50 Bio UV-visible spectrometer, measuring absorbance at

600 nm (OD_{600}). Sigma-Aldrich and EMD provided antibiotics. [^{14}C]-sodium citrate was purchased from Sigma-Aldrich and PerkinElmer. All radiation counts were detected by a PerkinElmer liquid scintillation analyzer tri-carbon 2900 TR. Scintillation fluid and scintillation vials were purchased from Fisher Scientific. Antibodies for western blots were purchased from Abcam. TargeTron Gene Knockout Kit was bought from Sigma-Aldrich

Chemical transformations of vectors into *E. coli*

The following is an outline for the transformation procedure. Unless otherwise noted, all *E. coli* transformations were done in this manner:

An eppendorf tube containing approximately 500 μ L of a 1:1 mixture of cells in media and 20% glycerol was removed from the -80 °C freezer and was allowed to thaw on ice. 50 μ L were pipetted into six eppendorf tubes along with 5 ng/ μ L, 10 ng/ μ L, 25 ng/ μ L, and 50 ng/ μ L of DNA mixture, control A (cell only), and control B (other plasmid positive control). 4 μ L of DNA (from PCR or stock) was added to their respective tubes. Nothing was added to control A. In the case of PCR from transformations, template DNA from a freezer stock was added to control B. All tubes were iced for 30 minutes, followed by a heat shock in a 42 °C water bath for 45 seconds, and then iced for two minutes. 650 μ L of super optimized culture (SOC) media was then

added to each tube. The cells were incubated at 37 °C for 1 hour. Cells were streaked across LB plates with ampicillin (50 ng/ μ L) to screen for antibiotic resistance. Controls A and B were also streaked on LB plates with kanamycin (35 ng/ μ L). Cells were incubated at 37 °C for 1 hour. The plates were then flipped and kept at 37 °C overnight. Success was measured by cell growth after 16 hours.

If colonies grew after the transformation, they were picked and grown overnight. The DNA was then isolated and restriction enzymes were used to see if DNA fragments of the correct lengths were present.

Electrical transformation of vectors into *K. radiotolerans*

First, electrocompetent *K. radiotolerans* cells were made and stored at -80 °C until use. The vector was then dissolved in TE buffer. 40 μ L of electrocompetent cells were thawed on ice. 1 μ L of DNA was added to the cells, and the contents were pipetted up and down to mix. The mixture was incubated on ice for 15 minutes. The mixture was placed into a 1 mm gap electroporation cuvette. The cells were electrocuted at 2000 V for 4.0 msec. The cells were incubated on ice for 2 minutes, and then iced SOC with 2 mM CaCl₂ was added to the cells. The cells were incubated at 30 °C for two hours. 1 μ L, 100 μ L, and 250 μ L aliquots were streaked across TGY plates. The plates were incubated at 30 °C overnight.

Digestion of DNA.

To a 1.5 mL eppendorf tube was added: Isolated DNA (16 μL), NEBuffer (2 μL), two restriction enzymes (1 μL each). The DNA was allowed to incubate at 37 °C for 60 minutes (or optimum temperature for restriction enzyme if other than 37 °C). To this mixture was added 6X loading dye (4 μL), and the sample was ready to load onto a 1% agarose gel. All (24 μL) of the DNA from the digestion was added to the wells alongside a 2 log or 1 kb ladder. The gel was run at 70 V for 90 minutes and analyzed under ultraviolet light.

¹⁴C Radio flux assays.

There were sixteen samples run for each trial. 2.2 mL centrifuge tubes were used for each individual trial. 100 μL of resuspended cells were pipetted into each of the sixteen vials. Special care was taken to swirl the cell contents to establish a uniform cell density throughout the solution. Also, caution was taken to ensure that there were no air bubbles in the pipette tip and that the pipette was touching the bottom of the vial so the cells would not adhere to the side of the tube. 5 μL of calcium chloride (220 mM) was added to every vial except for 3, 7, 11, and 15, which acted as the free citrate controls. The vials were shaken for 8 minutes. The shaker was stopped and 5 μL of radioactive citrate (96 μM) was then added to vials 13-16, 9-13, 5-8, and 1-4, specifically in that order. The final

concentration of the citrate was $4.4 \mu\text{M}$ and the final concentration of Ca^{2+} was 10 mM. At $t = 53$ seconds, the shaker was stopped and 0.1 M cooled LiCl ($970 \mu\text{L}$) was quickly added to vials 1-4, and then added once more. Vials 1-4 were placed on ice. At $t = 1:53$, the same was done to vials 5-8. At $t = 2:53$, the same was done to vials 9-12. At $t = 4:53$, the same was done to vials 1-4. In some instances, a 20 minute experiment was set up where the stopping solution was added at $t = 0:53$, $4:53$, $9:53$, and $19:53$.

A water aspirator was set up for the washing of residual citrate. A piece of 2.1 cm diameter P81 grade filter paper was placed onto a crucible with perforated holes in the base. The paper was moistened with 0.1 M room temperature LiCl. The contents in iced vial 1 was pipetted onto the filter paper. The filter paper was then washed with 0.1 M cooled LiCl ($3 \times 970 \mu\text{L}$) and transferred to a 20 mL scintillation vial containing 5 mL of scintillation fluid. This was repeated for all the vials. The vials were taken to EHO to measure radiation counts.

^{14}C Radio flux assay with native *Kineococcus radiotolerans*.

K. radiotolerans colonies were picked and grown in TGY media for two days. A TGY plate was streaked and the remainder of the cells were spun down and concentrated cell stocks were made. For the radio flux assays, a colony was picked in 5 mL TGY and allowed to grow at $30 \text{ }^\circ\text{C}$ and 175 rpm for two days. 1 mL seed culture was

added to a 50 mL culture flask. The cells were allowed to incubate under the same conditions for 24 hours. The cells were then centrifuged (4000 rpm, 10 minutes, room temperature) and resuspended in 10 mL of 50 mM PIPES buffer, pretreated with chelex, at a pH of 6.5. The cells were centrifuged and resuspended twice more under the same conditions.

In order to maximize the production of Cit_{Kr}, *K. radiotolerans* was grown in different media. First, TGY with 25 mM citrate was tested against minimal media with citrate as its only carbon source. TGY with 25 mM citrate was also tested against the same media, but with the addition of 10 mM Ca²⁺, 1 mM Mg²⁺, 0.075 mM Fe³⁺. The last experiment run for testing the growth media was with TGY only, TGY with 10 mM Ca²⁺, TGY with 25 mM citrate, and TGY with 10 mM Ca²⁺ and 25 mM citrate. The media that worked the best for calcium citrate uptake was TGY only. Now that the best media for metal-citrate complex uptake had been established, radio flux assays were performed with all of the metals described below.

The citrate stocks and metal stocks were mixed in a 1:1 ratio. 220 mM barium chloride, 220 mM calcium chloride, 22 mM cobalt chloride, 0.075 mM ferric chloride, 22 mM manganese(II) chloride, 22 mM nickel chloride, 22 mM lead(II) chloride, 22 mM strontium chloride, and 22 mM magnesium chloride were added to the 96 μM citrate into separate vials. The molecules were allowed to complex for 10 minutes before any radio flux assays were performed.

Uptake studies in native *K. radiotolerans*.

The procedure for the radio flux assays were repeated with Ca^{2+} -citrate only. The assays were tested at pH values of 6.1, 7.0, and 8.0, which are all in the buffer range for PIPES. The experiment was done in triplicate.

Radio flux assays with native *K. radiotolerans* in minimal media.

The same radio flux assays were performed using Ca-citrate only, except the media in which they were grown was changed. An Actinomycetales minimal media (1 g/L ammonium sulfate, 0.5 g/L K_2HPO_4 , 0.2 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g/L $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, and 10 g/L of a carbon source (sodium citrate in this example)) was made. A total of four different minimal media were made: two using this recipe, one at a pH of 7.0 and the other at a pH of 10.0. The other two recipes were identical, but supplemented with 1 mM CaCl_2 .

Cit_{Kr} Gene Knockout.

In order to prove that metal-citrate complexes are transported by a single gene system, a knockout was desired.

Knockout was first attempted using the TargeTron Gene Knockout Kit. The Cit_{Kr} gene was inputted into a knockout calculator form Sigma-Aldrich. Primers were designed and are as follows:

IBS 5'-AAAAAAGCTTATAATTATCCTTACCGCTCGTCATCGTGCGCCCAGATAGGGTG-3'

EBS1d 5'-

CAGATTGTACAAATGTGGTGATAACAGATAAGTCGTCATCCTTAACTTACCTTTCTTTGT-3'

EBS2 5'-TGAACGCAAGTTTCTAATTTCCGGTTAGCGGTCGATAGAGGAAAGTGTCT-3'

The kit also included a universal primer that can be used for all gene knockouts. A PCR was set up using the manufacturer's instructions using their supplied template DNA designed for the knockout. The DNA was purified by gel electrophoresis using a 1% gel run at 70 volts for 60 minutes. The DNA was extracted, doubly digested with *Hind*III and *Bsr*GI, and a ligation was performed with a pre-linearized vector using the method and gene/vector ratios as described earlier. The DNA was transformed into *E. coli* and streaked onto LB plates containing 40 ng/mL of chloramphenicol. The plates were incubated at 37 °C overnight. Colonies were picked, grown, and the plasmid was isolated as previously described. The DNA was doubly digested using *Cla*I and *Hind*III, purified by agarose gel electrophoresis, and extracted by the methods previously described.

pUC57_*Kr*KO was purchased with *Cla*I and *Hind*III restriction sites on the 5' end and the 3' end of the desired sequence respectively. This contained the origin of replication sequence for *K. radiotolerans*, as well as the ErmE promoter sequence. The vector was transformed into chemically competent *E. coli*. The plasmids were isolated and

digested with *Cla*I and *Hind*III. A ligation was performed with this DNA sequence and the vector as described previously. The product was transformed into chemically competent *E. coli* and streaked onto LB plates containing 40 μ g/mL of chloramphenicol. These cells were incubated at 37 °C overnight. The DNA product was also transformed into electrocompetent *K. radiotolerans* and streaked onto TGY plates: one containing 40 μ g/mL of chloramphenicol and one containing 40 μ g/mL of chloramphenicol and 35 μ g/mL of kanamycin. These plates were incubated at 30 °C for two days.

No successfully transformed bacteria were noted, so a new approach had to be developed. A plasmid was created containing the necessary components for the gene knockout by combining the portions of the *K. radiotolerans* genomic DNA believed to contain the origin of replication and promoter sequences with the TargetTron intron fragment.

Chapter 3

Results and Discussion

Kineococcus radiotolerans radio flux assays

Figure 1 shows the results for the preliminary radio flux assays of *Kineococcus radiotolerans* with a variety of metal-citrate complexes.

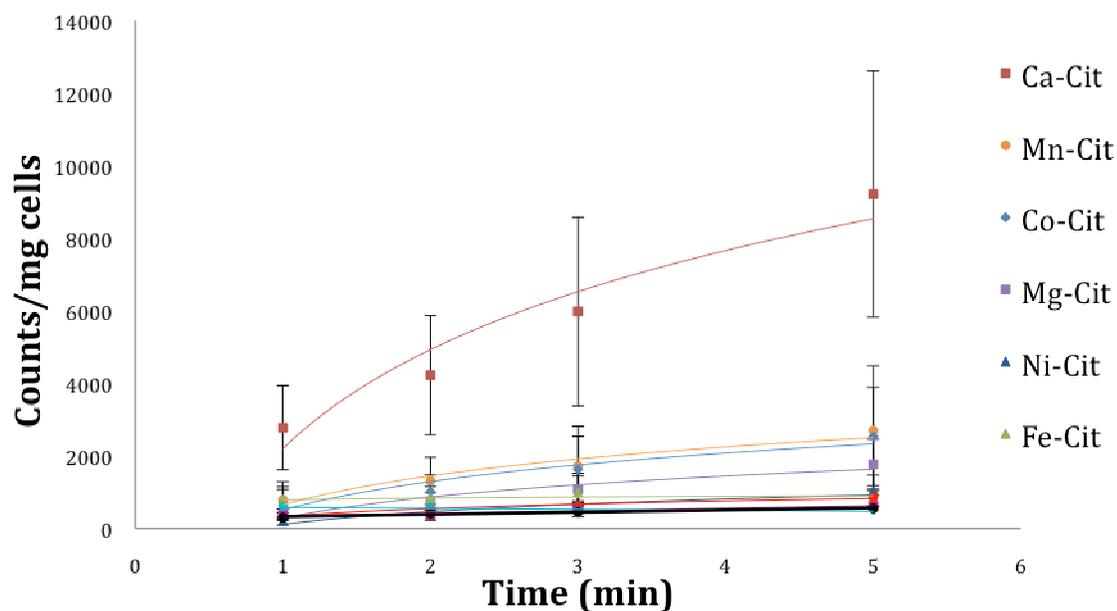


Figure 1. Metal-citrate uptake in *K. radiotolerans*.

The data in Figure 1 shows slight uptake of several of these complexes when compared to the free citrate control, but Ca-Cit is taken up by *K. radiotolerans* far more than any other metal-citrate

complex. The rate of calcium citrate uptake is also much greater than that of any other complex.

K. radiotolerans was originally found growing in toxic waste disposal sites, where the pH was close to 10. *K. radiotolerans* was therefore grown in TGY at normal pH (around 6.5) and TGY that had been altered to a pH of 10. Radio flux assays were performed to see how these changes affected Ca-Cit uptake. Figure 2 shows the metal-citrate uptake for cultures grown at neutral and basic pH values.

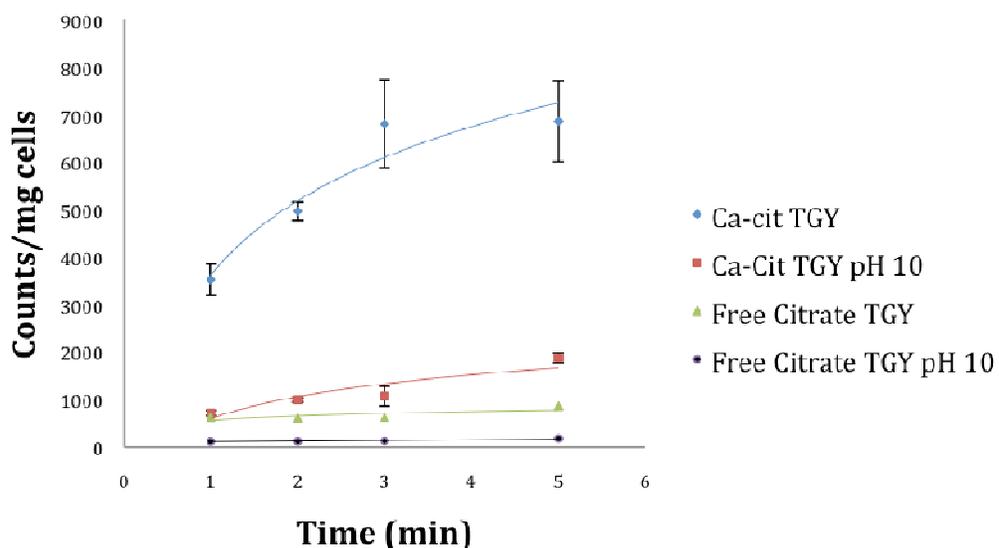


Figure 2. Uptake for Ca-citrate when the organism is grown at pH values of 7.0 and 10.0.

From Figure 2, it can be seen that when grown in TGY, the greatest Ca-citrate uptake occurs at pH 7. However, this trend is not observed when the *K. radiotolerans* is grown in a minimal media. These media were formulated to contain only citrate as a carbon source, and were either at pH 7 or pH 10. Additionally, Ca^{2+} was

added to one media at pH 7 and one at pH 10. Radio flux assays were performed and the results are shown in Figure 3.

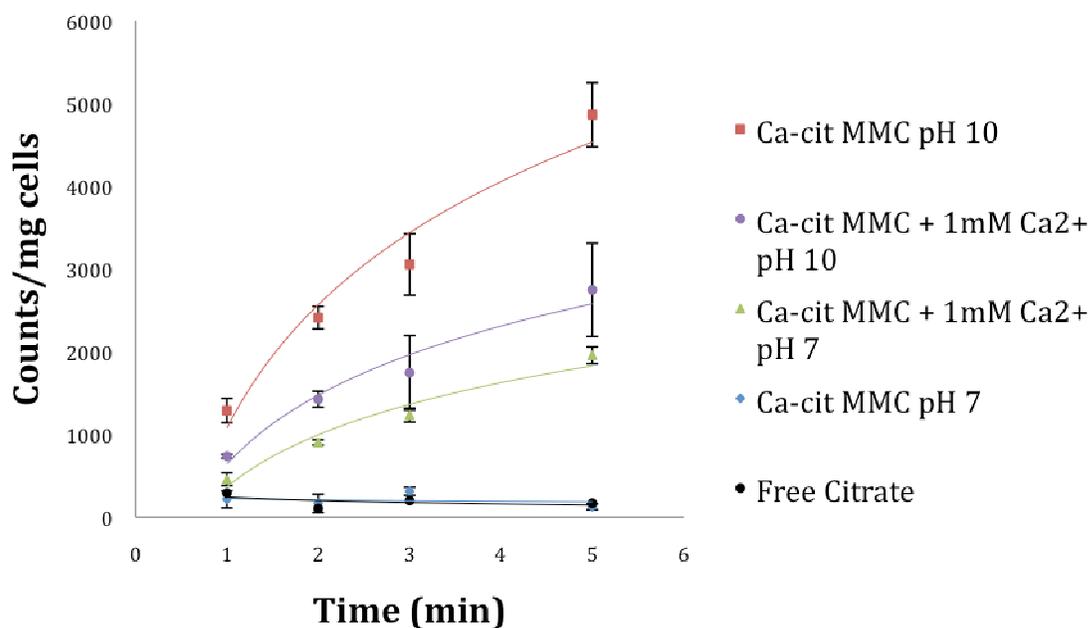


Figure 3. Ca-citrate uptake in *K. radiotolerans* in minimal media supplemented with sodium citrate or sodium citrate and Ca²⁺.

While it was expected that these results would follow the same trend as the cultures grown in enriched media, that was not the case. Here, the cells grown in MMC with no Ca²⁺ at a pH of 10.0 show the highest levels of uptake.

These results are consistent with the environment in which *K. radiotolerans* was first identified: a nuclear waste site with minimal nutrients, basic pH, and citrate present as the primary carbon source. It is likely that in the absence of enriched media and readily available glucose, *K. radiotolerans* uses citrate as a backup nutrient source. This would explain the increase in metal-citrate complex transport, as

more citrate is required for the cells to receive enough carbon to survive.

While previous experimentation certainly implies that Ca-citrate complexes are taken up by the organism, the trends for this uptake are not what would be expected. Experiments performed for 10 minutes tended to show a linear trend, so experiments were extended to 20 minutes in hopes of seeing the more logarithmic trend that was expected. Figure 4 shows the *K. radiotolerans* Ca-citrate radio flux assay run for 20 minutes.

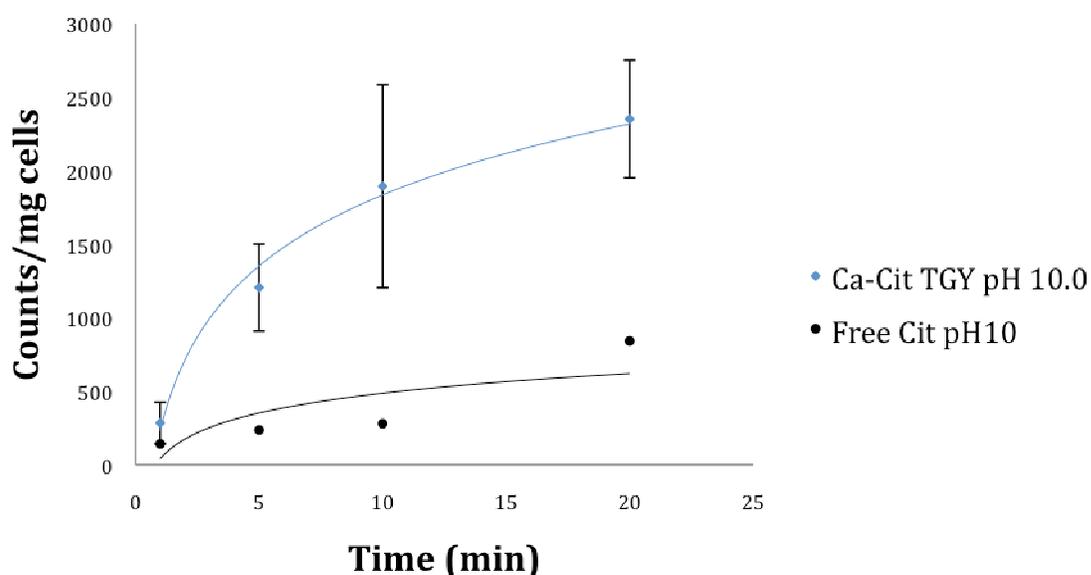


Figure 4. Free citrate and Ca-citrate uptake for *K. radiotolerans* for 20 minutes while grown at pH 10.0.

From this data the logarithmic trend is more clearly observable. Should the reaction be allowed to continue, the slope would most likely level off completely. Figure 5 shows a repeat of the Ca-citrate

transport for the minimal medias at different pHs, with and without Ca^{2+} , performed for 20 minutes.

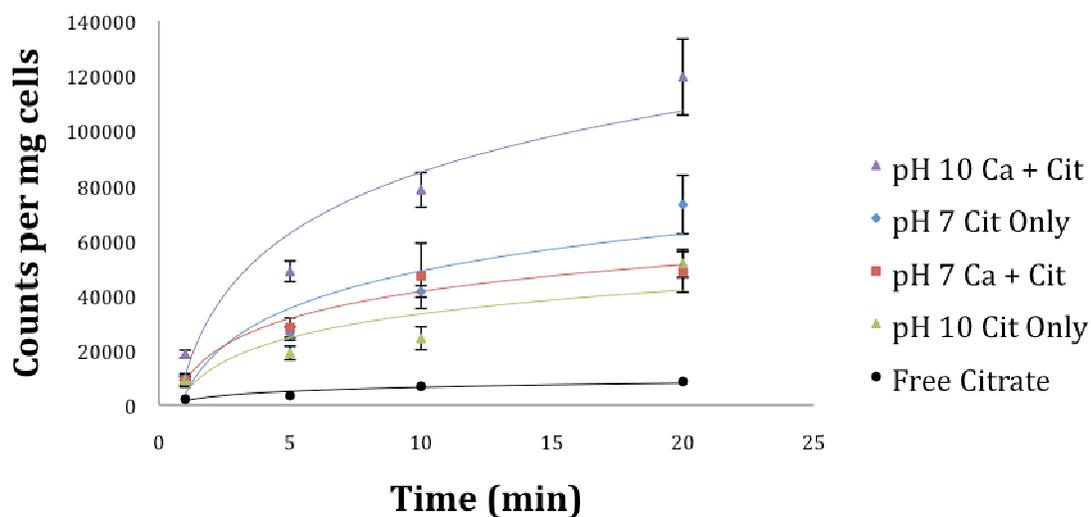


Figure 5. 20 minute Ca-citrate radio flux assay experiment conducted with cells grown in the minimal medias.

This data appears to confirm the previous hypothesis that in the absence of carbon sources like glucose, higher pH conditions are preferable for the function of the transporter.

Uptake assays in native *K. radiotolerans*

Figure 6 shown below shows further results for Ca^{2+} -citrate uptake at various pH values:

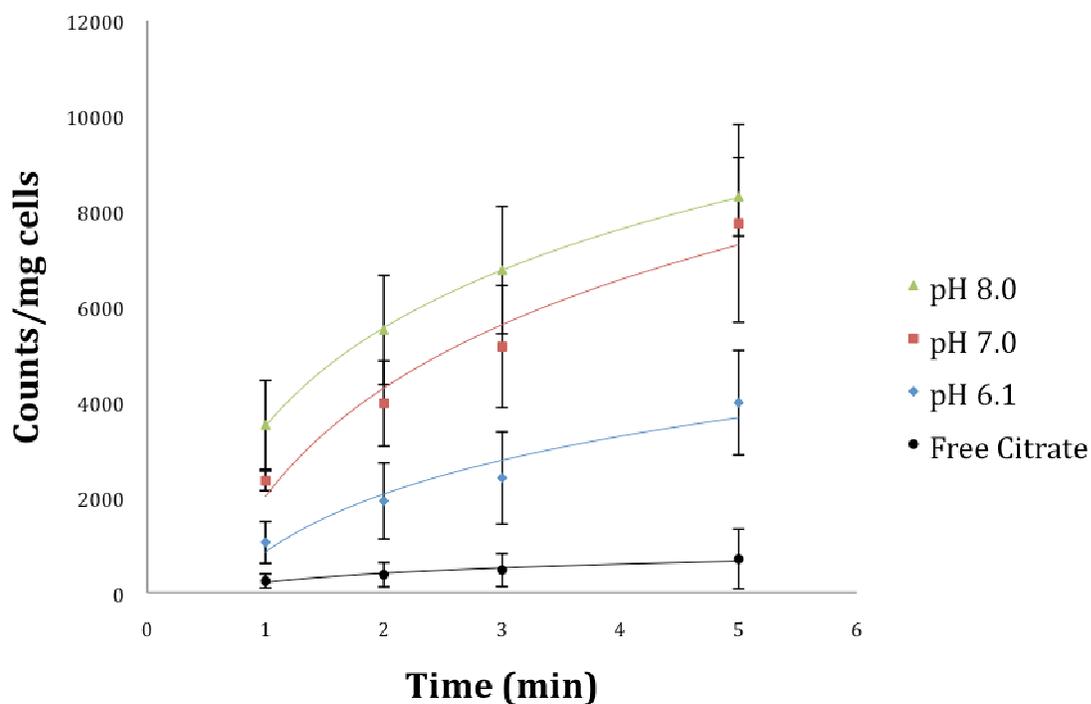


Figure 6. Ca^{2+} -citrate uptake at various pH levels.

This experiment shows a trend opposite of that previously observed in CitMHS members. While generally the transport increases as the pH of the solution decreases, in *K. radiotolerans* transport increases the pH of the solution increases.

This suggests that Cit_{Kr} may not utilize H^+ symport to transport the metal-citrate complexes. It is believed that this trend may show that instead the protein uses hydroxide antiport. If this is the case, Cit_{Kr} would be the first CitMOHA ever discovered. This theory is consistent with the elevated pH of the environment in which *K. radiotolerans* was discovered. In this case, an antiport protein is

likely an efficient solution. This is especially significant as *K. radiotolerans* has no way of taking up uncomplexed citrate.

Cit_{Kr} Knockout

Figure 7 shows the product for the amplification of the TargeTron intron sequence.

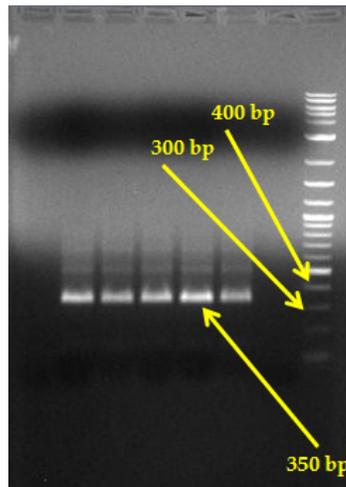


Figure 7. Doubly digested product for the intron segment for the knockout experiment.

The correct base pair size at 350 bp, and the product is seen at this size. After extraction, the fragment was ligated into the pre-linearized vector following TargeTron protocol and transformed. The confirmation of the product is shown in Figure 8.

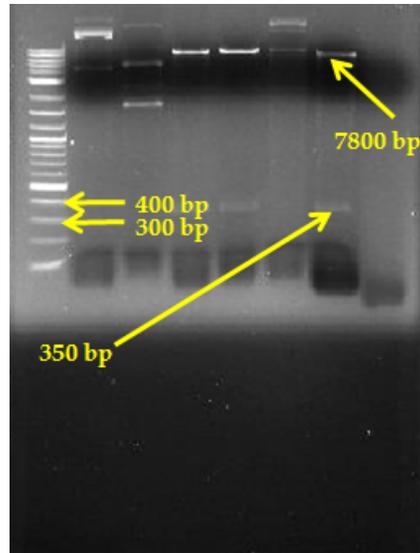


Figure 8. The 7800 bp band represents the linearized pACD4K-C and the 350 bp band shows the selected intron piece.

The DNA was sent for sequencing and the correct product was confirmed. Figure 9 shows the current plasmid construct, which has been shown to function in *E. coli*.

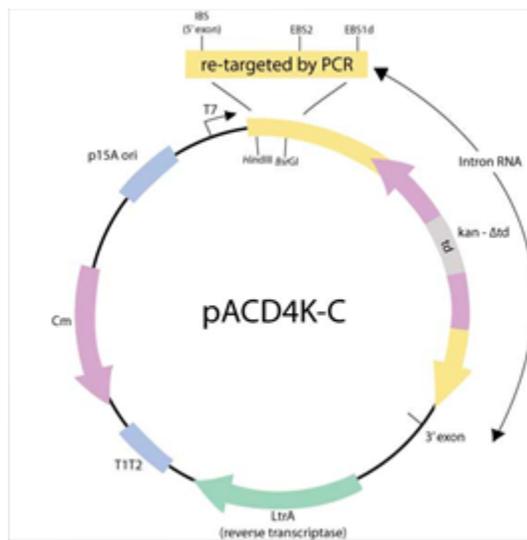


Figure 9. This shows the vector construct for generic genetic knockouts.

The T7 promoter sequence and the origin of replication sequence found in this vector are both designed for use in *E. coli*. Because the aim is to knockout Cit_{Kr} found in *K. radiotolerans* and this promoter is known not to function in this organism, more construction was needed before the vector could be used.

At present, there are no confirmed origins of replications or promoters for *K. radiotolerans*. One origin of replication has been hypothesized, and this gene was chosen for use in the modified plasmid. The promoter chosen was the ermE promoter, found in other organisms of *K. radiotolerans*'s order, Actinomycetales. These genes were constructed as shown in Figure 10.

```
TATCATCGATAAGCTCAAGGAGATGGCGCCCAACAGTCCCCCGGCCACGGGGCCTGCCA
CCATACCCACGCCGAAACAAGCGCTCATGAGCCCGAAGTGGCGAGCCCGATCTTCCCA
TCGGTGATGTCGGCGATATAGGCGCCAGCAACCGCACCTGTGGCGCCGGTGATGCCGGC
CACGATGCGTCCGGCGTAGAGGATCGAGATCTCGATCCCGCGAAATgccccctccggggga
ccggccggcgggggtgacgacgaggtcgtcccgcgcgacgggcccgtgagccgaccgtcccggggaac
acgtcccgggtggtcgatcccgggaacgcctccgcacgcccgtcaccggcccgtcgagggcccg
tcaagagcccgtccagggccctggggcagcccgcctcccggcgcaccacaccccaccgagg
gcccggcaccgcctcgcgggcccgtcccgcagggcctcctcccggcccgtccgaacacTTCTCCAC
AgTTGTCCACAcgctggggacagccaTGTGGACAAagatgtcctcagcggtggaacaactcggtgg
acaacgggtggatcccgcagcagggcgcctggggacggcgggtggacagcggcggcgcgcctccacct
ccctgtggaagcaggggtcgacgggtcctcaggcccgtccacaccacggaaacagggggcgaaccgc
ctctgacctgacgacgacaccgggTTGTCCACAgcgtgcacaagggctactacgacgatggaaccc
tctacgggtgaagttcgtcaacccacgtccactgtggaactggtggtggggacgggcagcccgtgagg
accgaggagcccgtgggggagaccgtcgcgttcgaacaagctgtgcgccgtcccgcagcctctgg
tacgggagagcagcagcggcggagccgcgcgggtggacgcagcgggtgcacggggccgggtcg
ctgtggaggcgcgtgcaccccgcgcggcgggtcctccactactgtcgggagcccggcccgcctgtcga
cgggcgggacgacggGATGCTGTTGTGGGCTGGACATCGTGCCGGTTGGTTAGGATCCAGCGGGGAA
TTGTGAGCGGATAACAATTCCCCTCTAGAAAAAAGCTTTATAA
```

Figure 10. The DNA sequence shows the additional unit added to the pACD4K-C vector. The green segment represents the *Cla*I restriction enzyme recognition sequence, red represents the origin of replication for *K. radiotolerans*, yellow represents the ermE promoter, and blue is the *Hind*III restriction enzyme recognition sequence.

This fragment was originally constructed into the pUC57 vector, with the origin of replication from *E. coli* still intact to simplify gene manipulation. The plasmid was isolated and doubly digested with *Cla*I and *Hind*III to remove the sequence of interest. The results are shown in Figure 11.

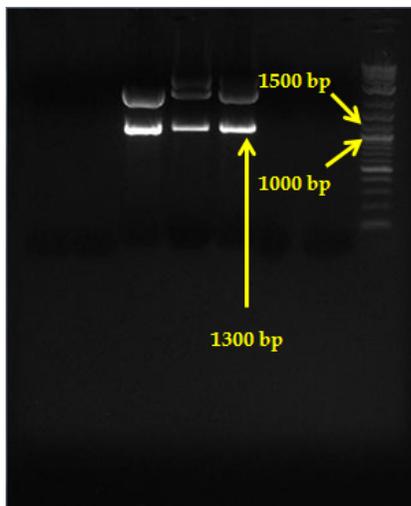


Figure 11. Verification that the new DNA sequence for the additional construct to the pACD4K-C vector is correct.

PACD4K-C was triply digested to prepare for another ligation.

The result is shown in Figure 12.

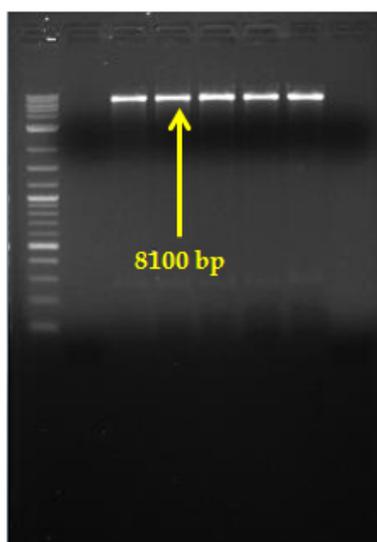


Figure 12. This shows the linearized pACD4K-C vector already containing the intron piece.

After linearization, the new DNA piece was ligated with the pACD4K-C vector. Once ligated, the DNA was transformed into *E. coli*. The plasmid was isolated and digested for verification. The result is shown in Figure 13.

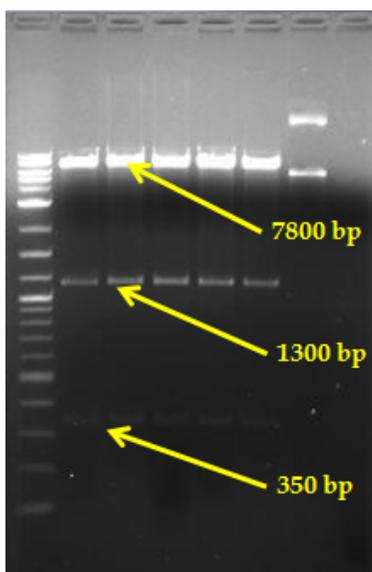


Figure 13. Vector-Dna construct triple digested with *Cla*I, *Hind*III, and *Bsr*GI. 7800 bp represents the vector, 1300 bp represents the new piece for *K. radiotolerans*, and 350 bp represents the intron fragment.

This data confirms that the full vector construct has been built correctly. The last step was to transform into *K. radiotolerans* in order to express the intron fragment and knock out the *Cit_{Kr}* gene.

The plasmid was transformed into electrically competent *K. radiotolerans* and streaked on TGY plates containing chloramphenicol or chloramphenicol and kanamycin. Growth on the chloramphenicol plates, would confirm successful plasmid insertion. Growth on the

plates containing both antibiotics, would confirm both plasmid insertion and gene knockout.

The bacteria did not grow on any of the plates. The process to make the electrically competent cells was altered to make the cells as susceptible as possible to DNA transformations. After these cells were made, the experiment was repeated, but still no growth was noted.

There are a number of reasons this could have occurred. It is possible the promoter and origin of replication did not function in *K. radiotolerans*. It is also possible that the *E. coli* optimized gene could not easily be read by *K. radiotolerans*, which has a much higher GC content. It is also possible that this difference in optimization led to the organism not having sufficient tRNAs to code for the antibiotic resistance genes.

In an attempt to correct these problems, a new plasmid is being constructed. This plasmid contains a new gene fragment extracted from the *K. radiotolerans* genome sequence shown in Figure 14.

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CAGCCGTGGACCGCGACCAGGACGTCGAACCTGGCCGCTGCGTCAATGACGACGACCTGGAGGCGTTTGCCTG

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Figure 14. New plasmid developed for the knockout. Purple = *XhoI* restriction recognition site, Blue = *ClaI* restriction recognition site, Green = Reverse coding GFP gene, Pink = Reverse coding LtrA reverse transcriptase gene, Red = Reverse coding chloramphenicol resistance gene, and Teal = Reverse coding intron piece.

It is believed that this fragment contains the native origin of replication and promoter for the organism. It is hoped that, should this plasmid function, it would generate a system that could be used in the future to express proteins in *K. radiotolerans*. The fragment currently contains the gene for GFP, which should make it easier to confirm the functionality of the plasmid in *K. radiotolerans*. The vector is currently being constructed.

Chapter 4

Conclusions

While progress has certainly been made in the study of CitMHS proteins, and especially of *Kineococcus radiotolerans*, more work must be done to gain the most thorough understanding of the function and future uses of this protein. This data shows the presence of a Ca-citrate transporter in *K. radiotolerans* and suggests that this transporter operates best in high pH, low carbon source conditions. This suggests that the primary purpose of this transporter is to provide the organism with an alternate carbon source when glucose is not available.

The results of these experiments also showed a different trend in uptake than had previously been noted in CitMHS members; while in these other proteins uptake tends to increase at lower pHs, in *K. radiotolerans* the uptake increases as pH increases. This could mean that this protein is not, in fact, a CitMHS member but a type of citrate-metal transporter never seen before: a citrate-metal/hydroxide antiporter. More work is required to confirm this theory, and if it is found to be true then research may reveal other members of this family.

Future work will also include more work on the gene knockout and use of the newly constructed plasmid to confirm loss of function. It is also believed that this plasmid can be used to perform gain-of-function experiments with CitMHS proteins that transport metal-citrate complexes other than Ca-cit.

Further work in this field could present a range of opportunities for the study and manipulation of CitMHS, and possibly CitMOHA, proteins. It is hoped that someday this research can lead to advances in the bioremediation of radioactive materials, and through study of other organisms with CitMHS proteins, methods of vaccination and detection for several dangerous diseases.

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Summary of Capstone Project

This project is focused on the study of the bacterium *Kineococcus radiotolerans*, an organism about which relatively little is known. This bacterium was first identified in radioactive uranium waste tanks and has been shown to be remarkably resilient to both high levels of radiation and high temperatures.

This organism was believed to contain a member of the CitMHS family of proteins. These proteins are imbedded in the cellular membrane and transport both hydrogen ions and metal-citrate complexes into the cell. Many cells use citrate as a carbon energy source, and metal ions are used in many cellular reactions, so it is not surprising that some organisms would develop a way to take up both simultaneously. These proteins have been identified in several organisms to date and there appears to be some variation in which metals are transported depending on the organism and protein in question.

The primary method by which this protein has been investigated is by radio flux assays. In these experiments, one of the carbons in citrate is replaced by radioactive carbon-14. The *K. radiotolerans* is then exposed to this citrate, both on its own and complexed with metal ions, for set amounts of time. If the organism has a metal-citrate transporter as hypothesized, the metal-citrate complexes are transported into the cell, while the free citrate is not. The amount of

radioactive citrate in the cells can then be quantified. Over time, the quantity of citrate in the cell should increase, then level off to generate a logarithmic curve.

The first set of experiments performed using this method exposed *K. radiotolerans* to a variety of metal-citrate complexes. Previous research had shown that the CitMHS transporters tend to transport only certain complexes, and our experiments showed that *K. radiotolerans* appears to take up calcium-citrate complexes, but very little of any other complex.

The next set of experiments performed focused on how different conditions of pH and available nutrients affected the function of the calcium-citrate transporter. When *K. radiotolerans* was grown in an environment where glucose was plentiful, the transport of the calcium-citrate was greatest at a neutral pH. In contrast, when *K. radiotolerans* was grown in an environment where citrate was the only available carbon source, transport was greater at a more alkaline pH. The latter set of conditions was nearly identical to those in which *K. radiotolerans* is naturally found.

These results lead us to several conclusions. It appears that when glucose is present, the calcium-citrate transporter is not very active; however, in situations where citrate is the only energy source available, far more calcium-citrate is taken up by the cell. It is likely

that in its natural environment with little to no glucose present, *K. radiotolerans* uses this transporter to survive.

While it was originally believed that the calcium-citrate transporter in *K. radiotolerans* was a member of the CitMHS protein family, the results of the pH experiments call this into question. In all previous experiments with CitMHS proteins, transport of the metal-citrate complexes has increased as the pH of the environment became more acidic. This is logical, as the protein transports hydrogen ions into the cell with each molecule of metal-citrate complex transported into the cell; in an acidic environment, there are more hydrogen ions to be transported. In *K. radiotolerans*, the transport of the calcium-citrate complex increased as the environment became more alkaline. This seems to indicate that this protein does not operate as a CitMHS protein does. Rather than transporting hydrogen ions and metal-citrate complexes into the cell simultaneously, the protein in *K. radiotolerans* appears to transport calcium-citrate complexes in while transporting hydroxide ions out. This mechanism also makes sense with *K. radiotolerans*'s native environment, which was very alkaline. This method of transport likely evolved as a response to these challenging conditions.

We hope to continue these experiments to confirm that the transporter in *K. radiotolerans* operates in this manner. We also hope

to extend our radio flux assays from 5 minutes to 20 in order to see an eventual leveling off of the amount of citrate in the cells.

We also hoped to identify the gene responsible for this transporter protein through experimentation. By comparing the genome of *K. radiotolerans* to organisms with CitMHS proteins, a sequence has been identified that is believed to code for the calcium-citrate transporter. In order to verify this, we hope to disable the purported gene sequence and see if this prevents the protein of interest from being generated.

In order to do this, we attempted a method that has been previously used to disable genes in other bacteria, like *E. coli*. This system uses a plasmid, a small circular piece of DNA, containing genes that can be manipulated to disable a gene of interest. This particular plasmid contains genes that allow it to function in *E. coli*, and previous research has shown that these genes do not function in *K. radiotolerans*. However, the equivalent genes for *K. radiotolerans* have not yet been identified, as research into this organism is in relatively early stages. In hopes of overcoming this problem, the plasmid was modified in such a way that it would function in another organism that is closely related to *K. radiotolerans*.

However, it appears that these attempts at modification were insufficient to allow this system to function in *K. radiotolerans*.

Currently, work is being done to generate an entirely new system to function in this organism.

Development of this system would allow for investigations of not only the calcium-citrate transporter in *K. radiotolerans*, but the metal-citrate transporters in other organisms. These transporters are believed to exist in extremely toxic organisms such as *Bacillus anthracis* (the bacterium responsible for the anthrax toxin) and *Staphylococcus aureus* (the bacteria that causes Staph infections). As these proteins are only found in bacteria, further insights into their structure and function could potentially lead to selective vaccines to protect against these serious infections. The same techniques used to generate vaccines could be used to generate test strips that could quickly identify the presence of dangerous bacteria. Manipulation of *K. radiotolerans* and a better understanding of the structure and function of its calcium-citrate transport protein could present a myriad of opportunities for better understanding organisms that present significant threats to human health.