GENE THERAPY: A NEW APPROACH FOR PREVENTING CALCIUM OXALATE STONES

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Dedication

This thesis is dedicated to my mom, Vera Lucia Maria Cruvinel Figueiredo and to my dad, Carlos Augusto de Carvalho Figueiredo. Thank you for all the love, unconditional support, dedication and encouragement you provided during this program. You are my heroes, my inspiration and my strength. Thanks for helping me succeed in several aspects of my career and my life.

Essa tese é dedicada a minha mãe, Vera Lucia Maria Cruvinel Figueiredo, e a meu pai, Carlos Augusto de Carvalho Figueiredo. Obrigada por todo amor, suporte incondicional, dedicação e força que vocês me deram durante essa jornada. Vocês são meus heróis, minha inspiração e minha fortaleza. Obrigada por me ajudar a sempre prosperar em todos os aspectos da minha carreira e da minha vida.

Table of Contents

List of Tables

List of Figures

Chapter 1 – Literature review

1. Literature Review

1.1 Calcium Oxalate stones

Urolithiasis is a general term referring to the causes and effects of stones anywhere in the urinary tract. Urolithiasis should not be viewed conceptually as a single disease with a single cause but rather as a sequel of multiple interacting underlying abnormalities. Thus, the syndrome of urolithiasis may be defined as the occurrence of familial, congenital, or acquired pathophysiological factors that, in combination, progressively increase the risk of precipitation of excretory metabolites in urine to form stones (i.e. uroliths). Several types of stones can be formed, and are classified based on their mineral components (1, 4).

The Minnesota Urolith Center has been analyzing uroliths for over 30 years and it has observed a dramatic increase in the prevalence of dogs and cats with calcium oxalate (CaOx) uroliths worldwide (1, 2, 3, 4, 5). They represent 40 to 45% of the samples analyzed in both species (2). In addition, over 90% of nephroliths and ureteroliths (kidney and ureter stones, respectively) are composed of calcium oxalate (12, 13). Urolithiasis incidence and composition have also changed in humans over the past five decades, with a substantial increase in the incidence of CaOx stones as well (6, 7). It's estimated that 10% of people will develop nephrolithiasis, and 70% of those will experience recurrences. Among them 80% of stones are calcium based, and 80% of those are calcium oxalate stone formers (11).

The driving force leading to urolith development is the supersaturation of urine with calculogenic substances. When calcium and oxalate reach supersaturation in the urine, calcium oxalate crystals spontaneously precipitate, crystallization takes place and crystals initiate aggregate formation (8, 9).

Epidemiologic studies support the hypothesis that in recent decades diets designed to minimize estruvite (magnesium ammonium phosphate – MAF) urolith formation may have increased the occurrence of CaOx stones. Diet-mediated urine acidification enhances the solubility of MAF crystals in urine, but promotes calcium oxalate crystalluria by inducing hypercalciuria (4). Increased excretion of calcium in the urine appears to be an important contributor to CaOx stone formation. However, it is not believed to be a significant risk factor in humans, since 24-hour urinary excretion of calcium is normal in some CaOx urolith formers and, in some nonurolith-forming humans, 24-hour urinary excretion of calcium is elevated (19). However, studies have shown that CaOx stone-forming dogs do excrete significantly higher levels of calcium than healthy non-stone forming dogs (20, 21).

Calcium homeostasis is achieved by a complex set of actions of parathyroid hormone (PTH) and vitamin D on intestines, kidneys and bones. Therefore, increased calcium levels can result from excessive absorption by intestines, decreased renal reabsorption and skeletal mobilization of calcium (9). The exact role of calcium in CaOx urolith formation in dogs and cats remains unclear, although combinations of familial, congenital and acquired pathophysiological abnormalities, such as primary hyperparathyroidism, vitamin D intoxication, osteolytic neoplasia and hyperthyroidism contribute to CaOx

urolithiasis in small animals (22, 23).

Nonetheless, hyperoxaluria due to increasing urine CaOx saturation is an important risk factor for calcium oxalate urolith formation. In humans, a vast number of epidemiologic studies confirmed that a small increase of oxalate concentration in the urine significantly enhanced the risk of CaOx urolith formation (23).

The precise cascade of events leading to CaOx stone formation is unknown. As a result, medical therapy to dissolve CaOx uroliths is currently unavailable, no treatment has been shown to be completely effective, contemporary therapies do not provide a long term solution and uroliths are commonly associated with lower urinary tract discomfort and potential life-threatening urethral obstruction. In addition, they are a common cause for ureteral obstruction and kidney failure. Although in concept surgical removal of upper urinary tract uroliths may restore kidney function, ureteral surgery has been associated with significant risks. In a study of 101 cats with calcium oxalate ureteroliths, the postoperative complication rate was 31% and the mortality rate was 18%. Chronic kidney failure present at the time of diagnosis remained following ureterolith removal (10).

Currently, voiding or retrograde urohydropropulsion, lithotripsy and surgery are the only options for removal of active CaOx uroliths (14). Although not common, CaOx urolithiasis associated with bacterial urinary tract infection should be treated. Uroliths that cause urethral obstruction should be retro-pulsed into the urinary bladder. This can be accomplished by retrograde urohydropropulsion, whereas voiding urohydropropulsion may be used to retrieve uroliths that have a diameter smaller than the diameter of the urethral lumen, as described by Lulich et al. (24). Another nonsurgical method is

lithotripsy, which breaks uroliths that are later collected by cystoscopic- or fluoroscopic-guided basket extraction of remaining fragments (9,25). Lastly, surgery can remove active ureteroliths and nephroliths, and nephrectomy is performed when kidneys are severely affected or nonfunctional (9).

Regardless of which treatment is chosen, CaOx stones are recurrent and because of that, removal of uroliths should not be the end point of therapy. During a study with thirty-three CaOx stone-forming dogs, 36% developed CaOx stones again after one year, 42% after two years, and 48% after three years (15). Thus, therapeutic strategies are needed to prevent formation and re-formation of CaOx stones.

1.2 Oxalate

Oxalic acid and/or oxalate salts are extensively present in animals, plants and microbes as a metabolic end product. In mammals, oxalate is generated endogenously (mainly in liver) from the metabolism of glycine, hydroxyproline, serine, glyoxylate, glycolate and ascorbate. As mammals are incapable of metabolizing it, oxalate is excreted via urine. Absorbed oxalate from the intestine also is excreted through the urine (29, 31).

It is not clear if the majority of urinary oxalate is derived from endogenous metabolic pathways or from the diet, although increased urinary oxalate appears to be sustained by an increased dietary load of oxalate and increased intestinal absorption. It was demonstrated that urinary oxalate concentration gradually declines in individuals that consume an oxalate-free diet and also that modifications in the diet can alter urine composition in dogs and humans (32, 33).

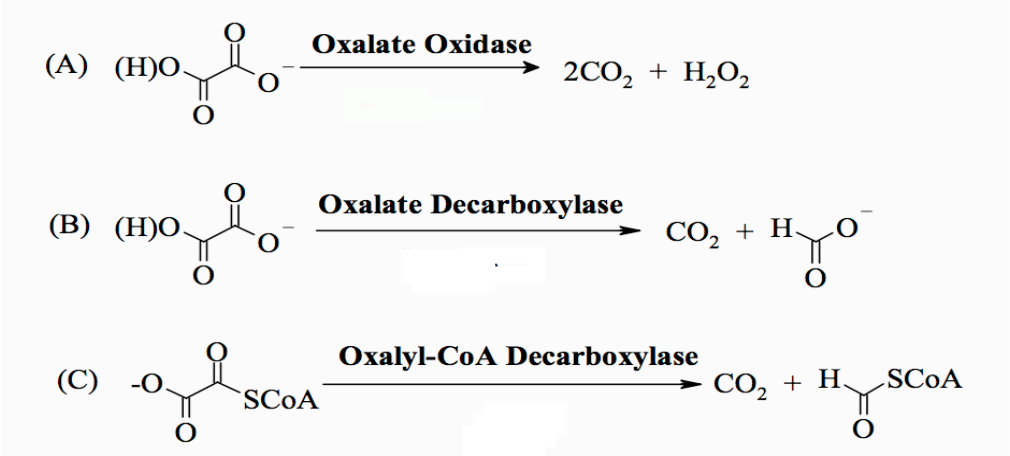
Dietary oxalate can be catabolized in mammalian species only through the action of oxalate-degrading bacteria. A popular example is the strict anaerobe, *Oxalobacter formigenes*, which colonizes the intestines of dogs, cats, rats and humans (26, 27, 28, 29, 30). Studies in rats showed that the intestinal microflora, in particular *Oxalobacter formigenes*, utilize oxalate in the gut, limiting its intestinal absorption and subsequent urinary excretion (16). In humans, colonization of the intestine with this bacterium showed reduction of urinary oxalate as well as plasma oxalate levels (17). Additionally, the presence of *O. formigenes* in the gut was associated with a 70% reduction in the risk for being a recurrent calcium oxalate stone former (18).

To summarize, enteric microbes metabolize oxalate and limit enteric oxalate absorption. It has been speculated that 50% to 80% of dietary oxalate is degraded by oxalate metabolizing bacteria in the gut (34).

1.2.1 Oxalate-degrading enzymes

There are three types of enzymes known to degrade oxalate in nature; oxalate oxidase (OxOx), expressed primarily in plants, although also isolated from the fungus *Ceriporiopsis subvermispora* (35, 36); oxalate decarboxylase (OXDC) and oxalyl-CoA decarboxylase (OXC), both of which are expressed in bacteria and fungal species (36).

Figure 1. Enzymes that catalyze the degradation of oxalate (adapted from Svedruzic (36)).



1.2.2 Oxalate Oxidase (OxOx)

OxOx is found in a wide variety of plants and converts oxalate to CO_2 and H_2O_2 (Figure 1). Its expression confers fungal disease resistance in crop plants, including *Amaranthus* leaves, beet stems and leaves, sorghum leaves, maize, oats, rice and rye (38), with the barley enzyme being the best described in plants (37) and *Ceriporiopsis subvermispora* in fungi (35).

Native OxOx is a glycoprotein that crystallizes as a hexamer, the biologically active form of the enzyme. It is hypothesized that the quaternary structure is responsible for the resistance of OxOx to degradation by heat and proteases. Each monomer of OxOx contains manganese (Mn), which supports the observation that specific enzyme activity is correlated with increasing Mn content, where oxalate binds on the catalytic site of the protein (35, 36, 37). Native OxOx exhibits an optimal enzyme activity at pH 4.0 (37).

The protein was shown to have a 65.5-kDa subunits (400-kDa hexamer) with a K_m for oxalate of 0.1mM and a K_{cat} of 88^{s-1}. The enzyme is located in membrane-bound vesicles that are exported to the periplasmic and extracellular space (39).

Oxalate oxidases belong to the cupin protein superfamily (35), which is divided into two broad groups: the monocupins (including OxOx and germin-like proteins) and the bicupins (including OXDC), which are twice the size of monocupins. However, sequences of OxOx from *C. subvermispora* resemble those of bacterial bicupin OXDC instead of the plant monocupin OxOx (known as germin). For that reason, fungal OxOx is a new class of OxOx (35, 36).

1.2.3 Oxalate Decarboxylase (OXDC)

OXDC catabolizes oxalate directly to formate and CO_2 (Figure 1). It was first described 50 years ago in the basidiomycete fungus *Collybia (Flammulina) velutipes* (36, 40). Subsequent studies have reported OXDC in other fungi, such as *Aspergillus niger, Sclerotinia sclerotiorum, Agaricus bisporus*, and in at least one bacterium, *Bacillus subtilis*. While the expression of OXDC in fungi is induced by oxalate, it is also induced in the bacterium by acidic pH (e.g. 5.0) (40,41).

All OXDCs are bicupins that require Mn as a co-factor, bound to both cupin domains, which is the same Mn-binding sequence motif that is present in OxOx. However, OXDC also requires dioxygen in the catalytic mechanism, being inactive when mixed with substrate (oxalate) under anaerobic conditions (36, 41, 42).

Fungal OXDC can be both an intracellular and extracellular enzyme, being secreted by hyphal cells. In the case of *B. subtilis*, the protein is exclusively a cytosolic enzyme (36, 41). The protein from *B. subtilis* has a K_m of 15 mM and a K_{cat} of 54 s^{-1} whereas in *F. velutipes* the K_m is 4.5 mM and has a K_{cat} of 166 μmol min^{-1} mg^{-1}, i.e. lower than *B. subtilis* (42).

Current models of enzyme evolution suggest that OXDC might have evolved from OxOx by gene duplication. OXDC has a quaternary structure composed of two homotrimers and each trimer resembles the OxOx hexamer (36).

1.2.4 Oxalyl-CoA Decarboxylase (OXC)

Oxalate degradation in bacteria is first mediated by oxalyl-CoA decarboxylase (OXC), which converts oxalyl-CoA into formyl-CoA and CO_2 (Figure 1). In order to complete oxalate metabolism by OXC, the activity of formyl CoA transferase (FRC) is required, since FRC transfers CoA from formyl CoA to oxalate and forms Oxalyl CoA. Both are essential to metabolize oxalate to formate and CO_2, along with a membrane-bound, formate/oxalate antiporter (encoded by the oxlT gene), importing oxalate into the cell and exporting formate. OXC is a thiamin-dependent enzyme that is constitutively expressed in *Oxalobacter formigenes*, an obligate anaerobe that solely depends on oxalate for ATP synthesis (36, 43).

Although the genes coding for OXC, FRC and OxlT play a role in the same function (oxalate metabolism), they are located in different operons, driven by independent promoters and terminator sequences (44). Therefore, as oxalyl-CoA decarboxylase uses a

very different and complex mechanism to metabolize oxalate, it will not be addressed further.

1.2.5 Heterologous expression of oxalate-degrading enzymes

Oxalate secretion by fungi provides advantages for their growth and is associated with plant pathogenesis. *Sclerotinia sclerotiorum* is an ascomycete that causes diseases in more than 400 plant species, including economically important crops such as tomato, soybean and lettuce. Transgenic lettuce lines containing the OXDC gene from *Flammulina velutipes* were produced, in order to confer resistance to these plants, by degrading the toxic oxalic acid (45).

Oxalate oxidase (OxOx) from *Ceriporiopsis subvermispora* was previously cloned and sequenced, and OXDC from *B. subtilis* was cloned into a recombinant *Lactobacillus plantarum* to degrade oxalate in vitro for future therapy against hyperoxaluria (46). In this study, *L. plantarum* constitutively overexpressed *B. subtilis* OXDC and was able to degrade more than 90% of the oxalate *in vitro* compared to 15% by the wild-type bacterium. (46). Additionally, our lab has engineered a probiotic strain, *E. coli* Nissle 1917 (EcN), to express heterologous *B. subtilis* oxalate decarboxylase capable of metabolizing oxalate *in vitro* (J. Gnandarajah, PhD thesis, University of Minnesota, 2007). In regards to the oxalate-degrading enzymes of *Oxalobacter formigenes*, stable expression of OXC and FRC genes in human embryo kidney 293 cells was reported, but there was no significant difference in oxalate degradation between samples and controls (47).

Finally, there are no published data on co-expression of all three genes (OXC, FRC, OxlT) in a heterologous host bacterium, nor any oxalate decarboxylase enzyme expression in a eukaryotic system.

1.3 Gene therapy

Gene therapy can be defined as a molecular-based tool used to transfer a gene that encodes a protein to functionally replace a defective or absent homolog in order to produce long-term expression. Gene therapy is becoming the medicine of the near future and there are currently several successful examples, as in the case of cystic fibrosis in humans and blinding retinal disease in dogs (48, 49).

Identifying a potential candidate for this approach in regards to preventing calcium oxalate stones, both in small animals and humans, is the first step to develop an effective therapy for this disease.

This study is expected to trigger further research focused on determining methods of gene transfer, identifying effective and safe delivery routes, prolonging transgene expression, and overcoming vector-mediated innate immune responses.

1.4 General hypothesis and objectives

Our *long-term research goal* is to develop safe and effective therapies to prevent stone recurrence in humans and animals. Here, our *principal objective* is to evaluate the feasibility of reducing oxalate concentrations by ectopic expression of oxalate-degrading

enzymes in a simple cell culture model of the kidney.

Our **hypothesis** is that enzyme expression and secretion from feline kidney cells (Crandall-Rees Feline Kidney, CFRK, cell line) will result in oxalate elimination from culture media. Efficient oxalate-degrading enzymes are not found in the mammalian genome, so bacterial and fungal enzymes were evaluated.

Objective 1. To clone different oxalate-degrading enzymes, since they might vary in efficacy of expression; Different sources of microorganisms able to degrade oxalate were used: *Bacillus subtilis* (OXDC), *Ceriporiopsis subvermispora* (OxOx) and *Flammulina velutipes* (OXDC).

Objective 2. To evaluate plasmid transfection into a feline kidney cell line, in order to characterize a transfection method optimized for this cell line.

Objective 3. To assess oxalate-degrading activity of transfected cells, by measuring oxalate and formate content in the media.

Chapter 2 - Cloning of oxalate-degrading enzymes

2.1 Introduction

The target oxalate degrading enzymes are monomeric, soluble proteins expressed in cytoplasm or secreted from the cell. Thus, they are expected to assume native, functional conformations that retain enzymatic activity in the extracellular environment of kidney cells. The proposed plan uses bacterial and fungal enzymes that are naturally secreted from host species maintained by University of Minnesota faculty, Dr. David Mc Laughlin and Dr. Robert Blanchette (Departments of Plant Biology and Plant Pathology, respectively).

2.2 Materials and Methods

In order to clone different oxalate-degrading enzymes, since they might vary in efficacy of expression, different sources of microorganisms (bacteria and fungi) able to degrade oxalate were used: *Bacillus subtilis* (OXDC), *Ceriporiopsis subvermispora* (OxOx) and *Flammulina velutipes* (OXDC) (Table 1).

Table 1. Oxalate-degrading enzymes' molecular features.

Species	Enzyme	Secreted	Signal sequence
Bacillus subtilis	OXDC	No	No
Flammulina velutipes	OXDC	Yes	Yes
Ceriporiopsis subvermispora	OxOx	Yes	Yes

Vegetative cultures of both fungi growing on agar in Petri plates were obtained from the Departments of Plant Biology and Plant Pathology (University of Minnesota). They were further grown in 2% malt agar stored in 18°C to 20°C for three weeks in order to increase the amount of mycelium for RNA extraction. Genomic DNA from *B. subtilis* was obtained from prior studies in our laboratory.

RNA from 15 mg of mycelium of each *F. velutipes* and *C. subvermispora* were extracted using the RNeasy Plant Mini Kit (Qiagen®) and cDNA synthesis was performed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems®).

Open reading frames (ORF) sequences were amplified by PCR (High Fidelity Polymerase - Clontech®) using primers designed based on sequences in GenBank. Amplification was performed in a GeneAmp PCR system 2400 (Perkin Elmer) with activation at 98°C for 5 minutes, followed by 35 cycles of denaturation at 98°C for 10 seconds, annealing at 55°C for 20 seconds and extension at 72°C for 1 minute and final elongation at 72°C for 5 minutes. Amplicons were resolved by agarose gel electrophoresis and visualized by ethidium bromide staining. Target DNA sequences were purified using QIAquick PCR Purification kit (Qiagen®) and recombinant plasmids were generated using the Infusion HD Cloning kit (Clontech®), which fuses DNA fragments and linearized vectors by recognizing a 15 bp overlap at their ends (Figure 2).

Two different mammalian expression vectors were used: pSG9M (52) and pSecTag by Invitrogen® (Figure 3). Among our genes of interest, fungal species contain a signal peptide (SP) sequence encoded within the gene, while the *B. subtilis* enzyme does not.

The pSG9M plasmid does not contain a plasmid-encoded leader sequence for guiding the protein into the secretory pathway in the cell, while pSecTag contains an IgK signal sequence for protein secretion. Therefore, these recombinant plasmids were generated based on the presence or absence of a signal peptide sequence in the vector and in the ORF.

Figure 2. Infusion cloning method (Infusion HD Cloning User Manual – www.clontech.com).

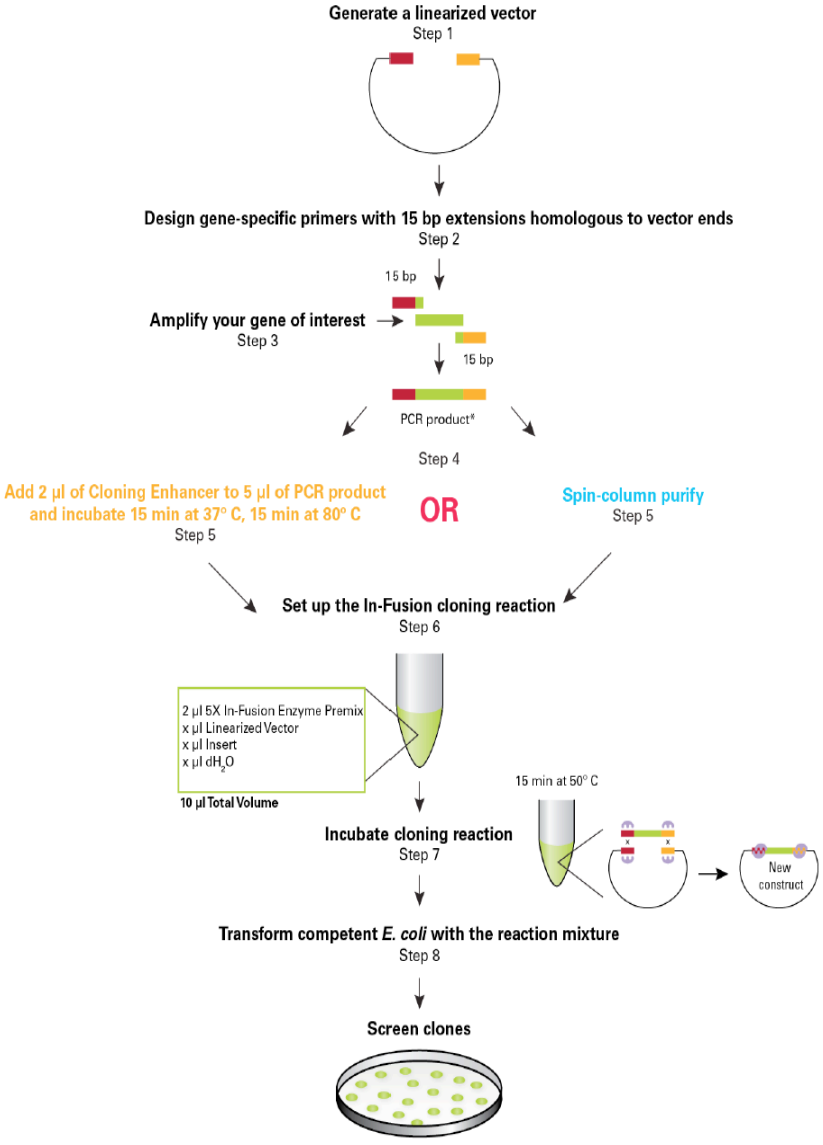
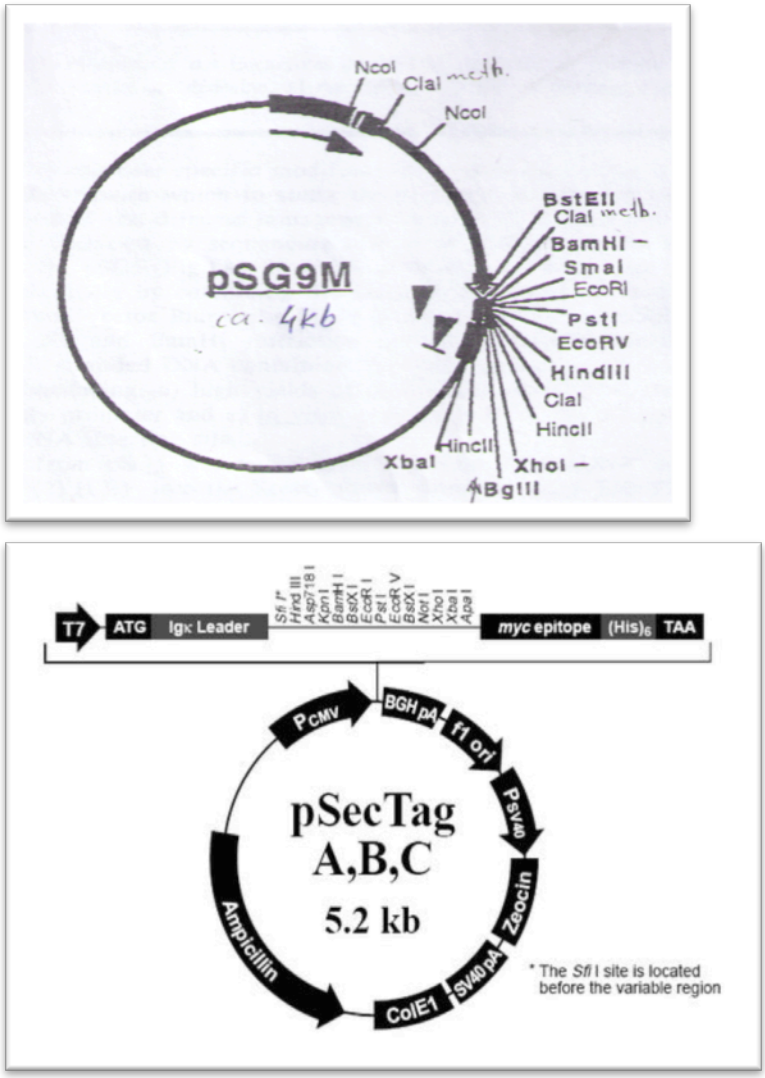


Figure 3. pSG9M and pSECTAG vectors.



Primers were designed based on published sequences and also constructed to overlap 15bp of target sequence and 15 bp of vector region (Table 2).

Plasmids were transformed into *E. coli* XL10 Gold cells and transformants were screened on Luria-Bertani (LB) agar plates containing vector-specific antibiotic (ampicillin). The following controls were included: competent cells without plasmid growing on plates without antibiotic (positive control for viability), and with antibiotic (negative control for

endogenous resistance), transformation with intact vector (positive control for transformation) and a linearized vector (negative control) and plating on antibiotic-containing plate. Clones were screened again by colony PCR and DNA from positive clones was sequenced. Fifteen colonies of each plate were screened for each of the samples. DNA fragments within the pSecTag-plasmid were amplified using primers targeting the vector T7 promoter (forward: TAATACGACTCACTATAGGG) and the BGH polyadenylation signal (reverse: TAGAAGGCACAGTCGAGG). DNA fragments within the pSG9M-plasmid were amplified using primers targeting the beginning and ending of the multiple cloning site region (MCS), as follows, forward: CGGTTACCAACATGGAACAA and reverse: CGAGGAAGCGGAAGAGTCTA (Figure 3).

Each positive colony was further grown in 5 ml of LB and ampicillin for 8h at 37°C with vigorous shaking (around 300 rpm). Then, 100 ul of starter culture was inoculated into 100 ml of LB and ampicillin at 37°C for 12 to 16h with vigorous shaking. Cells were then harvested by centrifugation and the EndoFree Plasmid Kit (Qiagen®) was used to purify recombinant plasmids from *E. coli* cells, in order to remove bacterial endotoxins that are released during the lysis step and that can further influence transfection of DNA into cultured cells.

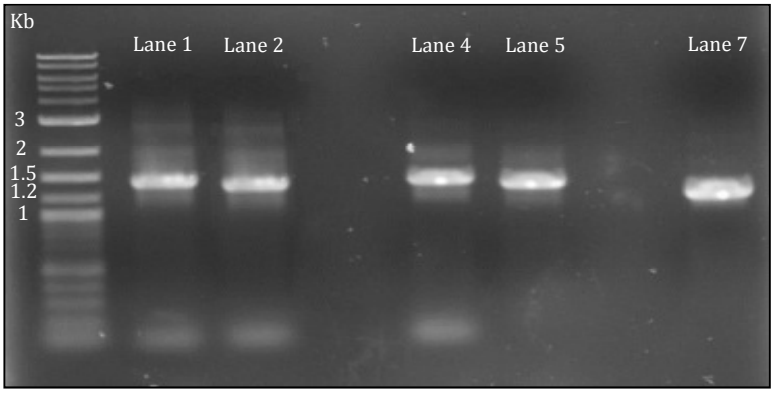
Table 2. List of primers used for amplification of target sequences.

Host / Enzyme / Vector	Sequence
B. subtilis / OXDC / pSG9M / Forward	CCATGAATTCTAGTGGAAATGACATTCCGCAGCCAAT
B. subtilis / OXDC / pSG9M / Reverse	GGATCGGGCCCCCCCTCTTTACTGCATTTCTTTTTCA
B. subtilis / OXDC / pSecTag / F	TTGGTACCGAGCTCGAATGACATTCCGCAGCCAAT
B. subtilis / OXDC / pSecTag / R	TTGTTCGGGCCCTCCTTTACTGCATTTCTTTTTCACT
F. velutipes / OXDC / pSG9M / F	CCATGAATTCTAGTGGATTCAACAACTTCCAACGTCT
F. velutipes / OXDC / pSG9M / R	GGATCGGGCCCCCCCTCGTTCACAGGACCAACAACA
F. velutipes / OXDC / pSecTag / F	TTGGTACCGAGCTCG TTCAACAACTTCCAACGTCTGC
F. velutipes / OXDC / pSecTag / R	TTGTTCGGGCCCTCCGTTCACAGGACCAACAACAGTC
C. subvermispora / OxOx / pSG9M / F	CCATGAATTCTAGTGGAAACGAGAAGCTCGTTT
C. subvermispora / OxOx / pSG9M / R	GGATCGGGCCCCCCCTCATCTGAGGCGACAACGAAT
C. subvermispora/OxOx/pSecTag / F	TTGGTACCGAGCTCGAACGAGAAGCTCGTT
C. subvermispora/OxOx/pSecTag / R	TTGTTCGGGCCCTCCATCTGAGGCGACAAC

2.3 Results

ORFs from all organisms were successfully amplified (Figure 4), and confirmed to be specific by sequencing.

Figure 4. PCR analysis of ORFs. PCR products; Lane 1: OXDC (1341bp) *F. velutipes*; Lane 2: OXDC (1281bp) *F. velutipes* (signal peptide – SP not included); Lane 4: OxOx (1418bp) *C. subvermispora*; Lane 5: OxOx (1358bp) *C. subvermispora* (no SP); Lane 7: OXDC (1158bp) *B. subtilis*.



PCR was performed in 15 colonies from each recombinant plasmid in order to check if the genes were correctly inserted within the vectors. All positive colonies were confirmed by PCR (Figure 5 and 6) and sequencing, except the clones related to *C. subvermispora* (OxOx), which resulted only in a few colonies, that did not contain the target ORF.

Figure 5. Colony PCR of OXDC from *B. subtilis* (lanes 1 and 7 – 1158 bp) and *F. velutipes* (lanes 2, 3, 4, 8, 9 and 10 – 1341 bp) within pSG9M vector (2log ladder).

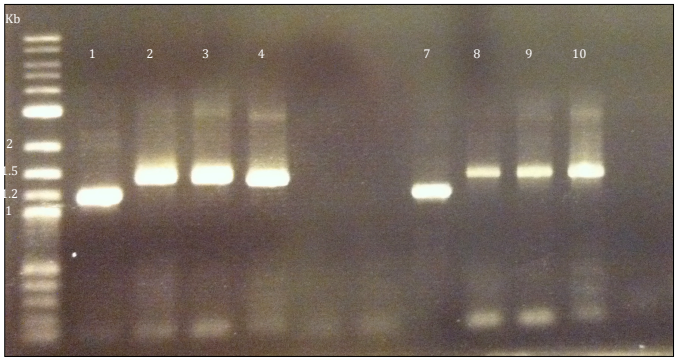
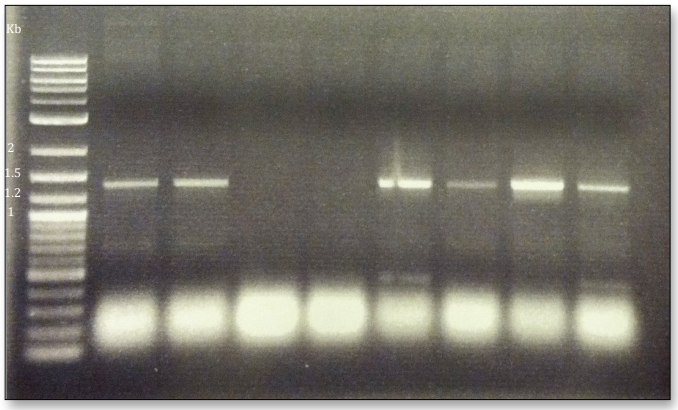
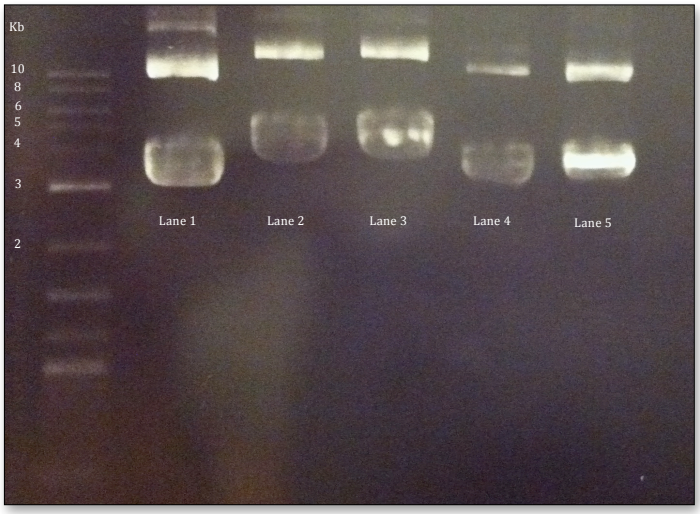


Figure 6. Colony PCR of OXDC from *B. subtilis* (1446 bp) within pSecTag vector - all lanes.



Successful recombinant plasmids were purified using the EndoFree Plasmid Purification Kit (Qiagen®) and samples were analyzed by agarose gel electrophoresis (Figure 7).

Figure 7. Gel electrophoresis of endotoxin-free plasmids (lane 1: pSecTag pure - 5.1kb, lane 2: pSecTag and OXDC from *B. subtilis* – 6.2kb, lane 3: pSecTag and OXDC from *F. velutipes* – 6.4kb, lane 4: pSG9M and OXDC from *B. subtilis* – 5.1kb, lane 5: pSG9M and OXDC from *F. velutipes* – 5.3kb).



2.4 Discussion

Enzymes able to degrade oxalate have potential applications, including production of transgenic plants that resist fungal disease, bioremediation, and treatment of hyperoxaluria. Excess of oxalic acid is toxic for all organisms and mammals are not able to degrade oxalate.

In our study, eukaryotic expression vectors pSecTag and pSG9M were used to clone OxOx and OXDC from bacteria and fungi. Both vectors contained a CMV (Cytomegalovirus) constitutive mammalian promoter for high-level expression and a c-myc epitope for detection with an anti-myc antibody. Secreted expression is the key function of pSecTag, since it also contains a signal peptide sequence derived from the mouse Ig kappa-chain mRNA for efficient secretion of recombinant proteins.

The well-known oxalate-degrading enzyme of *Oxalobacter formigenes* consists of three subunits, of which the catalytic component is a multiple membrane spanning protein, and exceptionally difficult to express in an active form (43, 44). Therefore, this work was focused on expressing oxalate-degrading proteins from other sources that might be more suitable for protein engineering and expression.

OXDC from *F. velutipes* has been successfully expressed in lettuce, tobacco and tomato, conferring resistance against the fungal infection of *Sclerotinia sclerotiorum*, a microorganism that produces copious amounts of oxalic acid (45). Similarly, OXDC from *B. subtilis*, was constitutively expressed in *Lactobacillus plantarum*, to degrade oxalate under *in vitro* conditions (46). Additionally, oxalate oxidase from barley (*Hordeum vulgare*) were cloned and tested in an *Escherichia coli* expression system, although the protein was not active (37). On the other hand, substantial amounts of active OxOx were obtained by expression in *Pichia pastoris* (53).

In summary, we have identified and isolated genes already known to be associated with oxalate degradation. They were cloned and recombinant plasmids were constructed using two different mammalian expression vectors, which were later amplified by bacteria in order to make large quantities of it. However, recombinant oxalate oxidase plasmid from *C. subvermispora* was either not taken by bacteria or unsuccessfully cloned, since colony PCR did not show presence of the gene, leaving oxalate decarboxylases from *F. velutipes* and *B subtilis* to be transfected and expressed by mammalian cells, in order to identify the best gene candidate for future gene therapy techniques.

Chapter 3 – Transfection optimization into Crandall Rees Feline Kidney (CRFK) cell line

3.1 Introduction

OXDC is a prokaryotic gene expressed in fungal and bacterial species and it is uncertain if OXDC can be expressed in eukaryotes. We constructed an eukaryotic expression system using CRKF cells, in order to check if this cell line can gain the capacity of oxalate metabolism by the heterologous expression of OXDC, becoming a promising new therapy for treating and preventing hyperoxaluria in small animals by targeting kidney-specific cells. In order to evaluate plasmid transfection and to characterize the best method optimized for this cell line, plasmids were purified and transfected into CRFK by different methods.

3.2 Materials and Methods

CRFK cells were maintained in Dulbecco's Modified Eagle Medium (Gibco® DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin (100 μg ml^{-1} and 100 U ml^{-1}, respectively) at 37°C in 5% CO_2.

Various transfection techniques, including lipofection (Lipofectamine 2000 – Life Technologies®), cationic polymers (Xfect Transfect Reagent – Clontech®) and nucleofection (Lonza®**)** were compared. Experiments used transiently transfected cells and all plates contained positive and negative controls. Positive control was used with a GFP-expressing plasmid and examination by fluorescence microscopy, and bisbenzimide staining of nuclei to determine transfection efficiency. The negative control was untransfected cells.

Transfected cells were detected by immunofluorescence (IF). Cells were fixed in 4% methanol-free paraformaldehyde in PBS for 20 min. Cells were rinsed 3x with PBS and permeabilized with 0.1% Triton X-100 for 10 min. Cells were then blocked with 3% BSA for an hour at room temperature. Primary antibody (mouse anti c-myc monoclonal antibody from Life Technologies®) was diluted 1:1000 and incubated with cells for an hour at 37^0C. After washing cells twice with PBST, Alexa Fluor 488 goat anti mouse IgG (Life Technologies®) was diluted 1:1000 in PBST and added with cells for 45 minutes at 37^0C protected from light. Lastly, staining of nuclei was performed by diluting 1:1000 bisbenzimide and incubating cells for 20 minutes at room temperature also protected from light. At this point, cells were washed with PBST and kept at 4°C until further analysis.

Cells were first transfected using the Xfect polymer according to the manufacturer's instructions. In this method, the negatively charged DNA binds to the polymer and the complex is taken up by the cell via endocytosis. 10,000 cells per well in a 24-well plate were transfected, and analysis was performed 48h after transfection. Several optimization steps were performed, including the amount of transfected DNA (0.5 to 2 ug), time of analysis (24h, 36h and 48h) and concentration of transfection polymer (0.1 to 0.6 ul).

The second method used was lipofection with Lipofectamine 2000(Life Technologies®), in which genetic material is injected into the cell by liposomes, which are phospholipid vesicles that can easily merge with the phospholipid bilayer of the cell membrane. Lipofection generally uses a positively charged (cationic) lipid to form an aggregate with

the negatively charged (anionic) genetic material. As with the Xfect reagent, this method was also optimized by adjusting concentrations of transfected DNA (0.5 to 5 ug), Lipofectamine reagent (2 to 5 ul) and time of analysis (24h, 36h and 48h). It was performed following manufacturer's instructions, using 10,000 cells per well in a 24-well plate.

The last method evaluated was nucleofection with a Nucleofector 2b device (Lonza®) and the solution V, as recommended by the manufacturer for this cell line. This tool uses a combination of electrical parameters, generated by the Nucleofector, that transfer the DNA directly into the cell nucleus and cytoplasm. 10,000 cells per well in a 24-well plate were used and several nucleofection programs were evaluated, all were reported as successful and had given satisfactory transfection efficiency rates, according to the manufacturer's webpage. Programs tested were T-020, T-019, V-013 and V-017; plasmid concentrations were 2, 5, 10 and 20 ug.

Transfection efficiency was calculated based on the total number of transfected cells (green fluorescence) divided by the total number of cells (nuclei staining).

ELISA protocol

Protein in the cell-free supernatant and lysate were detected by a "sandwich" enzyme-linked immunosorbent assay (ELISA), developed specifically to eliminate a need to purify the protein before measurement, simplifying the assay, and increasing the specificity and the sensitivity of the assay.

Briefly, wells of a 96-well plate were coated with a rabbit anti-myc antibody (Sigma Aldrich®) diluted 1:1000, in duplicate wells, 100 ul per well, and incubated 24h at 4°C. Plates were washed with PBST, pH 7.4, at room temperature in an ELx405 Microplate Washer® (Bio-Tek Instrument Inc. Winooski, VT). The plates are then washed 3X and blotted to remove excess liquid. 300 ul/well of Blocking Buffer (5% non-fat dry milk in PBST, pH 9.4) was added to each well and the plates were allowed to stand for 2h at room temperature. Plates were washed again and 100 ul of cell culture supernatant from CRFK cells transfected with oxalate degrading enzymes, grown in OptiMEM serum free media, was added and incubated for 1h at room temperature. Plates were washed again and secondary antibody was added (mouse anti-his monoclonal antibody, Novex®) diluted 1:1000 in NFDM, pH 7.4, was added to each well then incubated for 1h at room temperature. Plates were washed one more time and detection antibody was added (anti mouse IgG HRP conjugate (Bethyl®) diluted 1:10,000 in 5% NFDM in PBST, pH 7.4, and mixed thoroughly. 100 ul was added to each well and incubated for 1h at room temperature. Plates were washed for the last time and 100 ul of enzyme substrate was added, allowed to develop a blue color for exactly 15 minutes at room temperature, and finally 100 ul of 1M phosphoric acid stop solution was added. Plates were read at 450 nm on a Thermo Max Microplate Reader (Molecular Devices®, Sunnyvale, CA).

A positive control myc-tagged protein, the porcine corcovirus 2 replicase, was included in all plates at concentrations ranging from 5 to a 100 ng per 100 ul, along with untransfected supernatants and uncoated wells (negative controls).

Immunoblot

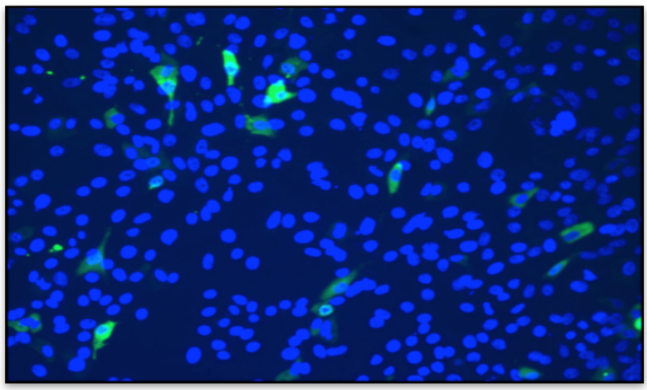
The total proteins of transfected cells were subjected to a 4-20% SDS-PAGE gel electrophoresis for 1 hour and then electro-transferred to a PVDF membrane (Millipore-FL®). The membrane was blocked with Odyssey Blocking Buffer (Licor®) for 1 hour at room temperature and then washed 3x with PBST. Incubation with mouse anti-myc monoclonal antibody (Life Technologies®) diluted 1:5000 with a labeling IRDye 680RD goat anti-mouse IgG diluted 1:1000, 0.1% Tween20 and 0.01% SDS was performed for 1 hour, shaking, at room temperature. Membrane was scanned on an Odyssey machine using a detection channel of 700 nm.

A positive control myc-tagged porcine circovirus 2 replicase was included, along with untransfected supernatants and lysates (negative controls).

3.3 Results

Crandall Rees feline kidney cells transfected with cationic polymers (Xfect reagent®) did not show satisfactory results. Transfection efficiency ranged between 5 to 20% for the GFP-containing plasmid (positive control) and less than 5% for samples (Figure 8). The results were obtained when added the maximum amount of plasmid DNA recommended (2 ug), analysis at 48 hours and using a concentration 0.6 ul of transfection polymer.

Figure 8. Transfected cells identified by green fluorescence (GFP-containing plasmid).



Lipofection also was not successful, showing less than 2% of transfection efficiency (Figure 9). However, when cells were transfected by nucleofection, transfection rates reached up to 95%. In this last method, efficiency increased as the concentration of plasmid transfected increased. Different amounts of plasmid were tested: 2, 5, 10 and 20 ug, being 20ug the optimal plasmid concentration (Figure 10). Among 4 nucleofection programs recommended by the manufacturer's website for this cell line, T-020 showed the highest transfection efficiency. Samples and controls were duplicates, 3 fields per well were analyzed for each and had their transfection efficiency averaged.

Figure 9. Lipofectamine-transfected GFP-containing plasmid CRFK cells.

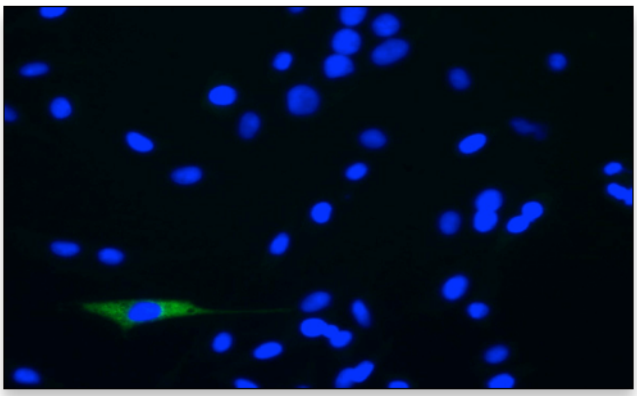
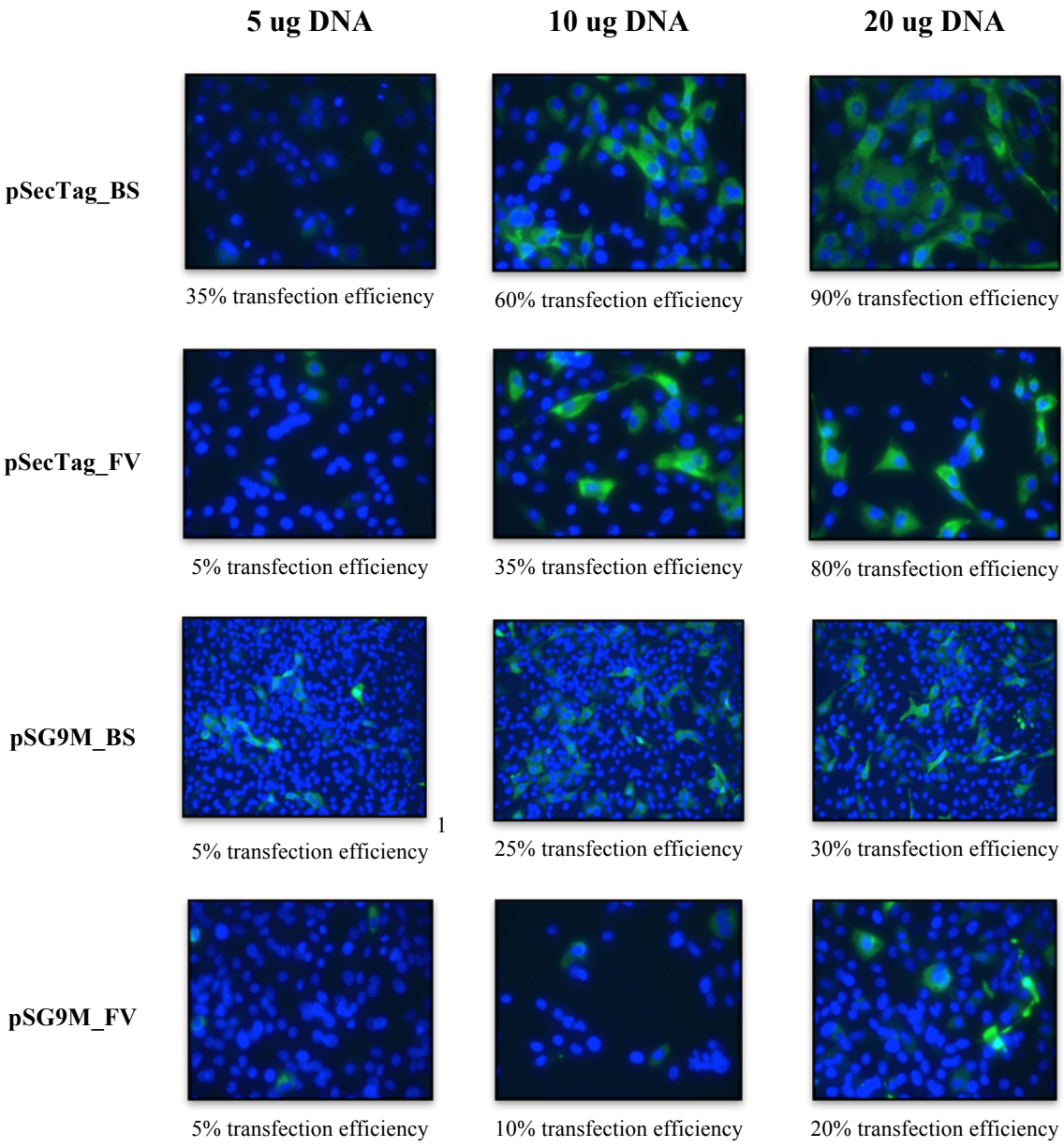


Figure 10. CRFK cells transfected by nucleofection. pSecTag_BS (OXDC from *B. subtilis*), pSecTag_FV (OXDC from *F. velutipes*), pSG9M_BS (OXDC from *B. subtilis*), pSG9M_FV (OXDC from *F. velutipes*) – Program T-020.



ELISA results showed high amounts of protein, both intracellular and extracellular, for both recombinant pSecTag cells. A standard curve was made based on the positive control protein myc-tagged from PCV (Figures 11 and 12).

Figure 11. ELISA results: standard curve based on the positive control – protein myc-tagged from PCV. Poly. (REP) – polynomial curve fitting;

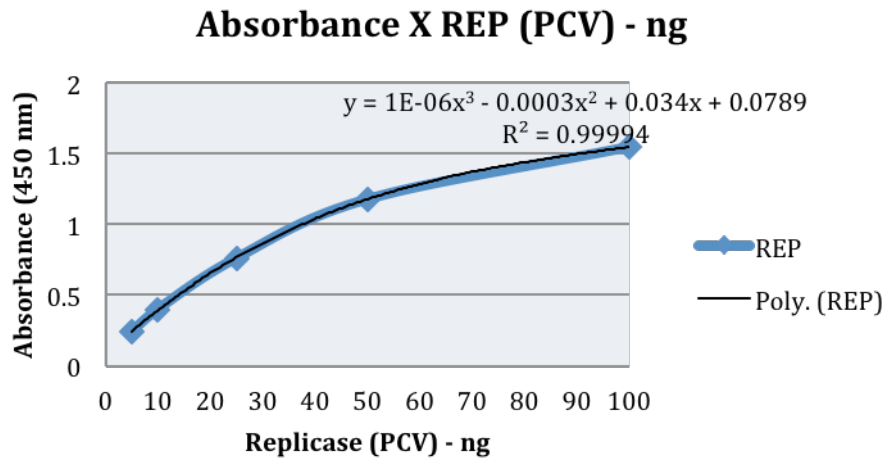
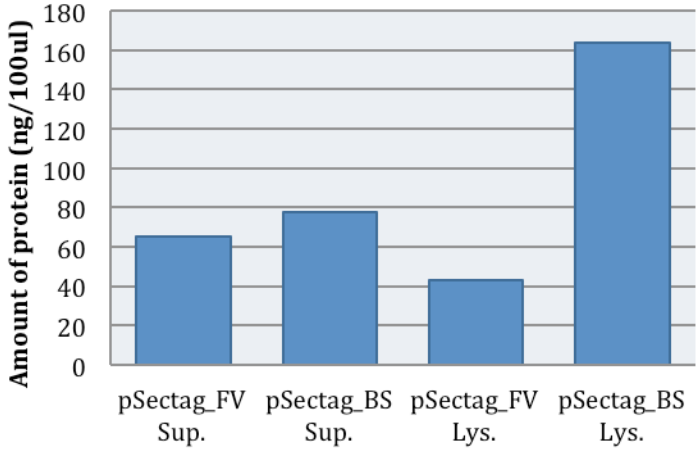
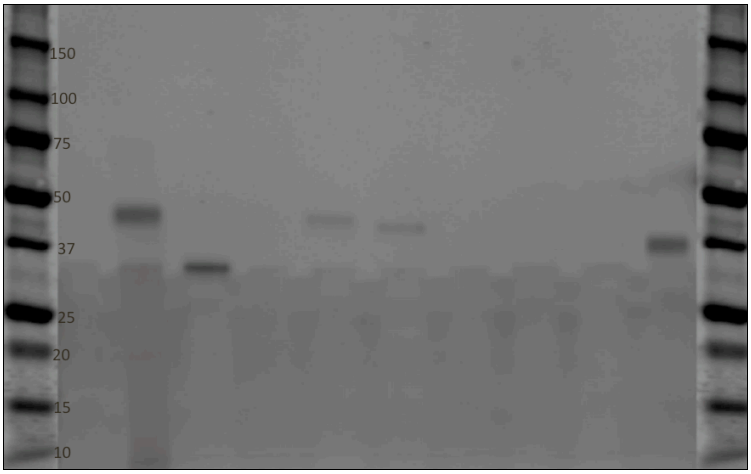


Figure 12. Amounts of proteins were determined by the standard curve.



The samples subjected to the ELISA assay, were also used to be evaluated by protein immunoblotting, which was performed using a mouse anti-myc monoclonal antibody. Supernatant and lysate total proteins of transfected cells were subjected to a 4-20% SDS-PAGE gel electrophoresis and the results showed bands corresponding to the predicted size of OXDC proteins (48kDa from *F. velutipes* and 43kDa for *B. subtilis*). The immunoblot included a negative control (untransfected cells) and positive control replicase. Expression of OXDC was detected in samples transfected with recombinant OXDC from both *F. velutipes* and *B. subtilis* (Figure 13).

Figure 13. Western Blot analysis. Lane 2: Protein supernatant of pSecTag_FV-transfected cells (OXDC); Lane 3: Cell lysate of pSecTag_FV-transfected cells (OXDC); Lane 5: Protein supernatant of pSecTag_BS-transfected cells (OXDC); Lane 6: Cell lysate of pSecTag_BS-transfected cells (OXDC); Lane 7: Protein supernatant of untransfected cells; Lane 8: Cell lysate of untransfected cells; Lane 9: myc-tagged protein control from PCV (REP).



3.4 Discussion

CRFK cells were isolated from the cortical portion of the kidneys of a 10-12 week old normal female domestic cat (50). There is no apparent loss of susceptibility of the cells to

selected viruses, not even after 200 passages, thus the cell line is extensively used in feline virus research (50). The experiments here used transiently transfected cells in order to investigate the short-term impact in protein expression. DNA was not integrated into host genome, and gene expression was temporary (24h to 96h).

Positive controls were performed with a GFP-expressing plasmid and examination by fluorescence microscopy, and bisbenzimide staining of nuclei to determine transfection efficiency. Endotoxins were removed from plasmid preparations, since some cell lines show reduced transfection efficiencies in its presence (54).

Transfection of CRFK cells was not previously reported, therefore multiple methods were evaluated. Cationic polymers (Xfect reagent®) and liposome-mediated transfection (Lipofectamine 2000 – Life Technologies®) did not show satisfactory transfection efficiency, although other kidney cell lines have been successfully transfected according to the manufacturer's website. Nucleofection, a technique that uses electricity to create transient pores in the cellular membrane to enable uptake of DNA into the cells, was highly efficient, resulting in up to 90% transfection when using pSecTag constructs. Transfection rates increased as the amount of plasmid was increased (Figure 10), which resulted in the use of 20 ug of plasmid for every experiment. However, the pSG9M vector-based test constructs showed a maximum efficiency of 30%. Thus, pSecTag_FV and pSecTag_BS were selected for further characterization since they gave higher protein expression and secretion levels.

A sandwich ELISA was developed to measure the amount of protein both in supernatant and lysate of transfected cells, by taking advantage of two antigenic epitopes (myc and

His tags) capable of binding to two antibodies (anti-myc and anti-His). Concentrations of recombinant proteins were determined by a standard curve based on a positive control protein (PCV2 replicase) with the same tags. Samples did not need to be purified prior to the assay, due to its high specificity. It was very useful for analysis of low protein concentrations in the presence of other cellular and serum proteins. In addition, protein immunoblot results also demonstrated that both recombinant OXDC proteins were being expressed and secreted by CRFK transfected cells.

However, it was still uncertain whether these recombinant proteins kept their enzyme activity as secreted proteins in a feline kidney cell line.

Chapter 4 – Oxalate-degrading activity of transfected cells

4.1 Introduction

In mammals, oxalate-degrading bacteria in the gut minimize the absorption of oxalate. Based on that, scientists have been trying to achieve a stable probiotic-colonization of these bacteria, although consistent reduction in urinary oxalate levels was not observed among different studies (55, 56).

In our approach, oxalate-degrading enzymes would be incorporated into mammalian kidney cells by gene therapy, giving them capacity to degrade oxalate and to reduce the levels of oxaluria. In order to accomplish that, two gene candidates that were successfully expressed and secreted by CRFK transfected cells had their protein function evaluated.

4.2 Materials and Methods

Proteins secreted from transfected cells had their oxalate-degrading activity evaluated by measuring oxalate content in the media through ion chromatography. Prior to that, different amounts of oxalate were added into the cells, in order to check for toxicity and to assess any changes in cell growth rates. Based on the results of a MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay, a non-toxic concentration of oxalate was determined and added into the samples in order to evaluate oxalate degradation over time.

A MTT assay was performed according to the manufacturer's instructions (Vybrant MTT Cell Proliferation Assay kit – Molecular Probes®) in a 96-well plate and 10,000 cells

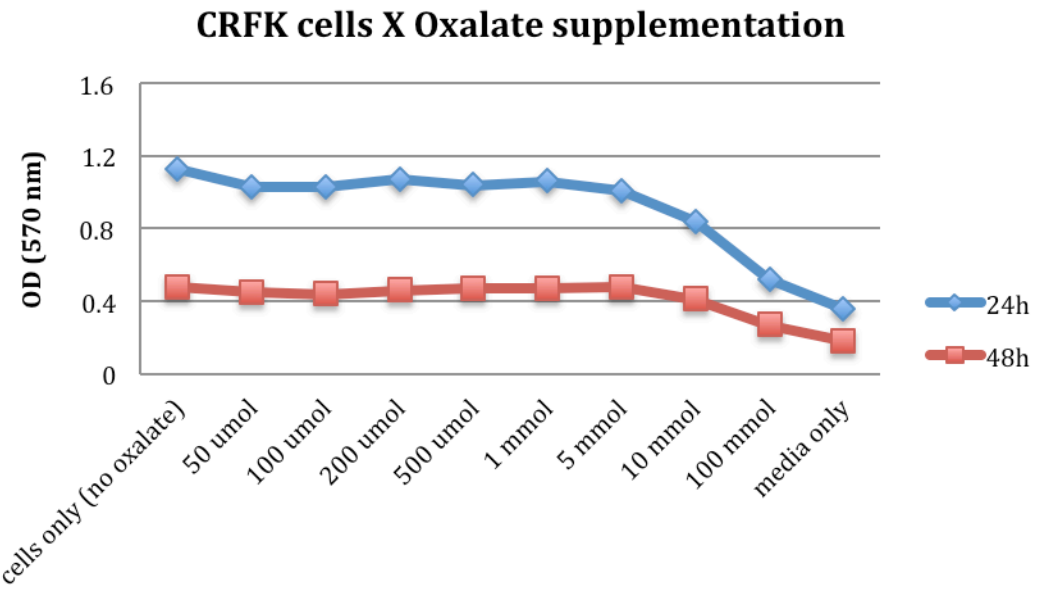
were plated in each well along with media (100ul). A 0.1 M solution of NaOx (sodium oxalate) was prepared, diluted in PBS and filtered to sterilize (0.2 um membrane filter). Serial dilutions were made and different concentrations of NaOx, ranging from 50 umol/L to 100 mmol/L were tested at 24h and 48h. Media only without cells (negative control) and cells only without NaOx (positive control) were included in each plate.

An amount of 5 mmol/L was added on both samples and controls at time of transfection. Oxalate and formate ion concentrations of cell supernatant was measured by ion chromatography (Department of Geology & Geophysics, University of Minnesota) 24h later. Samples were diluted with de-ionized water and analyzed on a Dionex ICS-2000 ion chromatography system consisting of an AS19 analytical column, ASRS 300 suppressor, AS40 autosampler, and integrated dual piston pump and conductivity detector. The eluant was generated by Reagent Free eluant generator system (Dionex, Sunnyvale, CA), which produced a variable concentration KOH eluant, regulated by Chromeleon control software. The control program used a comprehensive anion elution scheme.

4.3 Results

The results of MTT assay showed that at both time points there was a decrease in cell viability above 5 mM NaOx (Figure 14). Therefore, the following experiments were carried with 5 mM NaOx in the culture media.

Figure 14. Results of MTT assay – 24h and 48h.



To confirm that transfected cells were expressing a functional oxalate-degrading enzyme, cell culture media were supplemented with NaOx and both oxalate and formate concentrations were evaluated 24 hours later. Although results did not show a difference in oxalate levels between transfected cells and controls (Figure 15), formate levels confirmed enzyme activity and also revealed cellular oxalate production and metabolism in samples that were not oxalate-supplemented (Figure 16).

Figure 15. Ion chromatography results showing oxalate concentration among samples and controls.

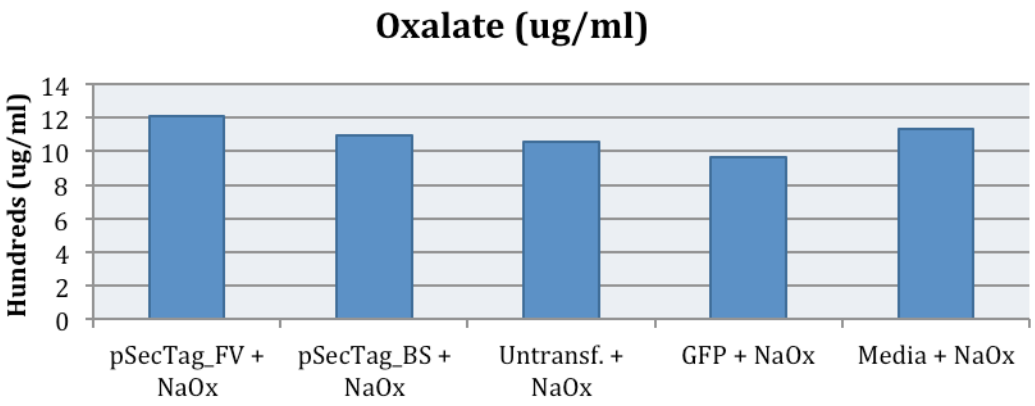
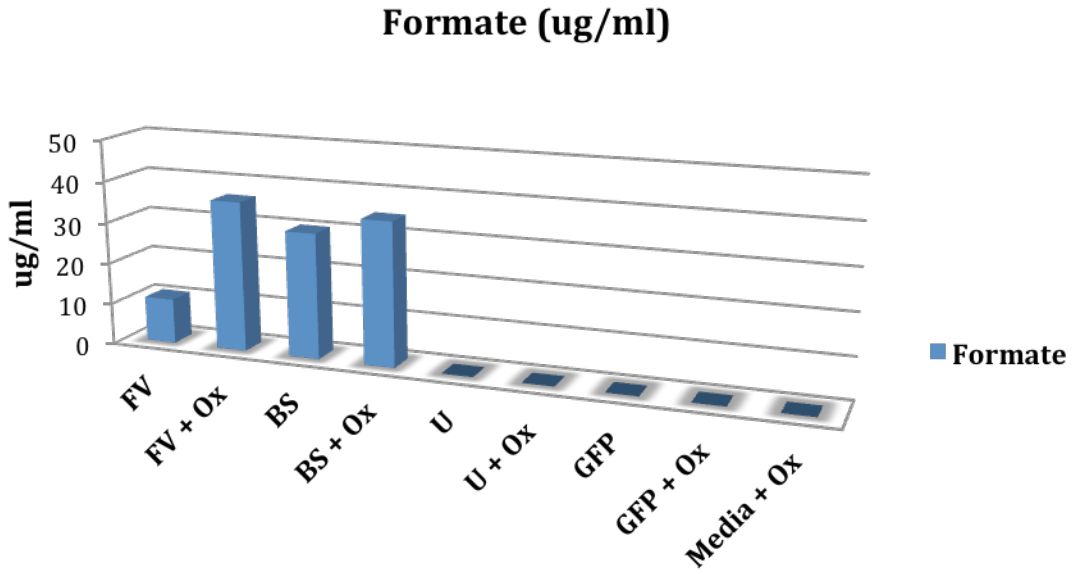


Figure 16. Ion chromatography results showing formate accumulation among samples and controls.



4.4 Discussion

The MTT assay is based on the conversion of MTT into formazan by living cells, which corresponds to the mitochondrial activity. In most cell populations, the total

mitochondrial activity is directly related to the number of viable cells. Therefore, this assay is broadly used to measure the cytotoxic effects of drugs and other chemical compounds utilized on cell lines in vitro. In this study, the addition of NaOx was only toxic to the cells on concentrations greater than 10 mmol/L, with no difference after 24 or 48 hours. Based on these results, an amount of 5mmol/L NaOx was added into samples and controls to be further compared by ion chromatography after 24 hours.

Ion chromatography provides high sensitivity and low detection limits (oxalate: 0.1 umol/L or 0.01 ug/ml – 1x dilution). Additionally, it is the standard method for quantifying oxalate, is relatively rapid, and requires no sample pretreatment other than dilution. Nevertheless, analysis of ion chromatography results revealed that recombinant OXDC, both bacterial and fungal, did not decrease the oxalate concentration in media compared to negative control GFP-transfected cells supplemented with the same concentration of NaOx. These results suggest that an excessive amount of oxalate might have been added to the cells and we were not able to detect any oxalate-degrading activity.

Since formate is the direct product of oxalate degradation by OXDC, its accumulation was also determined instead of oxalate disappearance for evidence of enzymatic activity (51). It was the first time ever reported that formate levels were used to evaluate OXDC activity. Results clearly showed that untransfected CRFK cells or GFP-transfected cells, supplemented or not with NaOx, did not produce any formate, whereas recombinant OXDC-transfected cells showed formate production. These results also confirmed our suspicion that cells basally produce a certain amount of oxalate – resulted of cell

metabolism, since OXDC-transfected cells and not NaOx supplemented, showed a certain amount of formate levels.

Finally, decreasing pH of the culture medium and/or adding oxalate to the cultures induces OXDC expression in many fungi and *B. subtilis* (36, 40, 41, 42). Regardless of the species of origin, OXDC requires oxygen for catalytic turnover, has optimal activity at acidic pH, is specific for oxalate as substrate, and contains a manganese (Mn) ion binding sequence, although it is not known if the Mn-binding sites participate in oxalate metabolism or in substrate recognition. In addition, OXDC was expressed in a foreign, mammalian environment. It is possible that post-translational processing or other processing functions in CRFK cells caused a reduction or loss of enzymatic activity.

In summary, further studies are needed in order to optimize enzymatic activity and to further characterize enzymatic activity. This result demonstrates the feasibility of a promising therapy and prevention tool against CaOx stones.

Chapter 5 – General discussion and conclusion

5.1 General Discussion

Accumulation of oxalate can lead to development and recurrence of CaOx urolithiasis. Treatment options for these patients are limited and do not provide a long term solution, hence new therapeutic approaches are still needed. We asked in this study, whether overexpression of OXDC from fungal and bacterial species can decrease the amount of oxalate in a mammalian cell culture system, as an in vitro model. We isolated the OXDC gene and studied its expression in a heterologous system, optimizing transfection methods and protein secretion. This is the first attempt ever reported to express an oxalate-degrading enzyme OXDC into a mammalian cell line. This study provides evidence that recombinant OXDC can be expressed, secreted and active in eukaryotic cells, which is an important step towards gene therapy approach for hyperoxaluria. However, further studies are needed, in order to optimize its activity.

5.2 Conclusion

A successful outcome is expected to directly benefit companion animals that suffer recurrent CaOx stone attacks that are refractory to current therapies and, after further refinement, gene therapy may become a front-line option. Additionally, this study will trigger additional research focused on determining methods of gene transfer, identifying effective and safe delivery routes, prolonging transgene expression and overcoming vector-mediated innate immune responses. This is a completely novel and promising approach that has potential for high impact on animal and human health.

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