Thesis for the Master’s degree in Molecular Biosciences
Main field of study in Molecular Biology

60 study points

Expression of the bacterial *ectA* gene in the chloroplast of *Chlamydomonas reinhardtii*

Anders Lunde

Department of Molecular Biosciences
Faculty of Mathematics and Natural Sciences
UNIVERSITY OF OSLO 12/2012
Acknowledgements

The master project was performed at the department of Molecular Biology at the University of Oslo in Professor Uwe Klein’s laboratory.

I would like to thank Professor Uwe Klein for his outstanding guidance, support, and supervision throughout the work that was performed. Thank you for the patience, understanding, and insight that has made the year in your lab both educational and fun.

A big greetings and thanks to my fellow master students at the Klein lab, Biruk, Pedro, and Ragnhild. I can’t imagine how it would have been to do a master project without you. We helped each other, and had a lot of fun together.

Additional acknowledgements goes to Anders Moen for performing MS experiments and teaching me about it, to Maria Luisa Salvador for testing the OectA vector plasmids, to Fabio Giulio Moratti for helpful comments on the project, and Gro Live Fagereng for allowing me to use her master thesis as an example.

Finally, thanks to my family and friends for all your love and support.

Oslo, December 2012
Anders Lunde
Summary

Ectoine is a small molecule that confers salt tolerance to organisms. It is synthesized in bacteria by the gene products of *ectA*, *ectB*, and *ectC*. Ectoine is used in medical, biotechnological, and cosmetic products.

The purpose of this project was to determine whether expression of the *ectA* gene in the chloroplast of the model alga *Chlamydomonas reinhardtii* would be sufficient for ectoine production.

Non-photosynthetic *Chlamydomonas* was transformed by microprojectile bombardment, using a plasmid vector with a chimeric *ectA* gene and a photosynthesis selection marker. In addition, a transformation vector with a codon optimized version of the *ectA* gene was designed, and used for bombardment. Transformants were analyzed by DNA and RNA blotting techniques. SDS-PAGE, and mass spectrometry were used to evaluate EctA accumulation. HPLC, and salt tolerance experiments were used to evaluate the accumulation of ectoine.

One chloroplast transformant was isolated for the non-optimized *ectA* gene that was about fifty percent homoplasmic. No transformant could be isolated with the codon optimized *ectA*. Transcript accumulation was detected for the *ectA* transformant, however at low levels. EctA protein accumulation could not be detected. HPLC did not identify any ectoine accumulation, and no increased salt tolerance of the *ectA* transformant was observed.

It is concluded that the current chimeric *ectA* gene is insufficient in supporting high levels of ectoine accumulation, probably because of the low transcript levels supported, and the lack of codon optimization. Further work is needed to improve the accumulation of EctA to higher levels.
Abbreviations:

ATP - adenosine-5'-triphosphate
bp - base pair
BSA - bovine serum albumin
cia - circa (approximately)
cm - centimeter
DEPC - diethylpyrocarbonate
dH₂O - distilled H₂O
DNA - deoxyribonucleic acid
DNase - deoxyribonuclease
dNTP - deoxynucleotide triphosphate
e.g. - exempli gratia (for example)
etc - et cetera (and other things)
g - gram
g - gravitational force
kb - kilobase pair
M - molar concentration
mA - milliampère
Mb - megabase pair
mg - milligram
mL - milliliter
mRNA - messenger RNA
N - normality
ng - nanogram
nm - nanometer
pmol - picomol
psaB - photosystem I subunit B
rbcL - rubisco large subunit
RNA - ribonucleic acid
RNase - Ribonuclease
UV-light - ultraviolet light
UTR - untranslated region
v/v - volume by volume
w/v - weight by volume
w/w - weight by weight
µg - microgram
µL - microliter
µCi - microcurie
# Table of Contents

ACKNOWLEDGEMENTS ........................................................................................................... 2  
SUMMARY .................................................................................................................................. 3  
ABBREVIATIONS ......................................................................................................................... 4  
TABLE OF CONTENTS ................................................................................................................. 5  

## 1.0 INTRODUCTION

1.1 BIOLOGY OF SALT STRESS .............................................................................................. 8  
   1.1.1 Salt tolerant organisms and their habitats ................................................................. 8  
   1.1.2 Salt survival strategies ............................................................................................... 9  
   1.1.3 Compatible solutes ................................................................................................... 10  
   1.1.4 Metabolic engineering of crop plants with compatible solutes .................. 10  
1.2 ECTOINE ........................................................................................................................... 11  
   1.2.1 Discovery and characterization .............................................................................. 11  
   1.2.2 Commercial interest in ectoine ............................................................................... 12  
   1.2.3 Ectoine in metabolic engineering .......................................................................... 13  
1.3 THE MODEL ORGANISM *CHLAMYDOMONAS REINHARDTII* ................................ 13  
   1.3.1 General features ..................................................................................................... 13  
   1.3.2 The *Chlamydomonas* chloroplast as a protein expression system .............. 14  
   1.3.3 Codon bias in the *Chlamydomonas* chloroplast ............................................... 14  
   1.3.4 *Chlamydomonas* chloroplast transformation ...................................................... 15  
   1.3.5 Transformation by microprojectile bombardment ............................................... 16  
   1.3.6 Chloroplast transformation with photosynthesis selection marker ................ 17  
1.4 ECTOINE PRODUCTION IN *CHLAMYDOMONAS* BY EXPRESSION OF *ECTA* IN THE CHLOROPLAST ......................................................................................... 18  

## 2.0 MATERIALS AND METHODS

2.1 WORK WITH *ESCHERICHIA COLI* .................................................................................. 21  
   2.1.1 Preparation of agar growth plates with ampicillin ................................................ 21  
   2.1.2 Preparation of competent *E. coli* TB1 cells (CaCl$_2$ method) ...................... 21  
   2.1.3 Transformation of competent *E. coli* ................................................................. 22  
   2.1.4 Growth of *E. coli* on agar plates with ampicillin ............................................ 22  
   2.1.5 Culturing of *E. coli* agar plate colonies ............................................................. 22  
   2.1.6 Storage of *E. coli* at -80 °C .......................................................... 23  
   2.1.7 Miniprep plasmid isolation from *E. coli* ............................................................ 23  
   2.1.8 Maxiprep plasmid isolation from *E. coli* ............................................................ 23  
2.2 WORK WITH DNA ......................................................................................................... 24  
   2.2.1 Agarose gel electrophoresis ................................................................................. 24  
   2.2.2 Purification of DNA fragments from agarose gel .............................................. 25  
   2.2.3 Quantification of DNA (dot spot) ............................................................... 25  
   2.2.4 Quantification of DNA by ultraviolet light absorption ..................................... 25  
2.3 SUBCLONING .................................................................................................................. 25  
   2.3.1 Plasmids .............................................................................................................. 25  
   2.3.2 Polymerase chain reaction (PCR) ...................................................................... 26  
   2.3.3 Restriction digestion of DNA ......................................................................... 27  
   2.3.4 Partial restriction cutting of DNA ................................................................. 27  
   2.3.5 Ligation ............................................................................................................... 27  
   2.3.6 Codon optimization of ectA ......................................................................... 28  
   2.3.7 Sequencing ........................................................................................................ 28
2.4 WORK WITH CHLAMYDOMONAS

2.4.1 Preparation of solid media for Chlamydomonas

2.4.2 Preparation of liquid media for Chlamydomonas

2.4.3 Culturing the Chlamydomonas photosynthesis mutant for transformation

2.4.4 Harvesting and plating of the Chlamydomonas photosynthesis mutant for transformation

2.4.5 Microprojectile bombardment

2.4.6 Recovery and selection of transformants

2.4.7 Photosynthetic growth of Chlamydomonas

2.4.8 Photosynthetic growth of Chlamydomonas with 2 % CO₂

2.4.9 Total DNA isolation from Chlamydomonas

2.4.10 RNA isolation from Chlamydomonas

2.5 ANALYTICAL METHODS

2.5.1 DNA and RNA blots

2.5.1.1 Preparation of radioactive probes (random primer labeling)

2.5.1.2 Hybridizing sample DNA and RNA with radioactive probe

2.5.1.3 Autoradiography with radioactive membranes

2.5.1.4 Aligning agarose gel photo to autoradiographic films

2.5.1.5 Slot blot

2.5.1.6 Southern blot

2.5.1.7 Northern blot

2.5.3 Mass spectrometry

2.5.4 High pressure liquid chromatography (HPLC)

2.5.5 Salt tolerance experiments

3.0 RESULTS

3.1 MAKING THE ECTA TRANSFORMATION VECTOR CONSTRUCT

3.1.1 ectA transformation vector overview

3.1.2 Subcloning of ectA

3.2 TRANSFORMATION AND SELECTION OF ECTA TRANSFORMANTS

3.2.1 Microprojectile bombardment and phototrophic selection

3.2.2 DNA slot blot

3.3 ANALYSIS OF TRANSFORMANT

3.3.1 Southern blot

3.3.2 Northern blot

3.3.3 SDS-PAGE

3.3.4 Mass spectrometry

3.3.5 High pressure liquid chromatography

3.3.6 Salt tolerance experiments

3.4 MAKING A CODON OPTIMIZED ECTA TRANSFORMATION VECTOR

3.4.1 Codon optimization of ectA

3.4.2 Subcloning of OectA

3.5 TRANSFORMATION AND SELECTION OF OECTA TRANSFORMANTS

3.5.1 Microprojectile bombardment and phototrophic selection

3.5.2 DNA slot blot

4.0 DISCUSSION

4.1 ectA transcript levels

4.2 EctA protein accumulation

4.3 EctA stability, activity and toxicity

4.4 Transformosome
4.5 SDS PAGE and mass spectrometry ......................................................... 59
4.6 High pressure liquid chromatography .................................................... 59
4.7 Salt tolerance experiment ......................................................................... 60
4.8 EctA substrate availability ....................................................................... 60
4.9 Optimized ectA ....................................................................................... 61

CONCLUSION .................................................................................................. 62
FURTHER WORK ............................................................................................. 62
APPENDIX 1 Graphical codon usage analysis ............................................... 64
APPENDIX 2 Mass spectrometry results ......................................................... 65
APPENDIX 3 Transformation vector sequences ............................................. 68
APPENDIX 4 Solutions and recipes ................................................................. 72
REFERENCE LIST .......................................................................................... 76
1.0 Introduction

1.1 Biology of salt stress

1.1.1 Salt tolerant organisms and their habitats

Surviving in salty environments is a challenge for cellular organisms. The amount of dissolved salts in their surrounding media directly affects their most precious molecule: Water. High levels of salts causes an osmotic effect that can empty cells of water, and thus kill them (DasSarma and DasSarma, 2001).

Although salinity is a serious challenge, a vast number of life forms have evolved to thrive in salty environments (DasSarma and DasSarma, 2001). Organisms that can live in water with high salt concentrations are designated either halophilic, or halotolerant. Halophilic species are those organisms that grow best at elevated salt concentrations, and their optimum growth can occur at anywhere from 1-30 % NaCl (w/w) (DasSarma and DasSarma, 2001). Halotolerant species are organisms that can support a certain level of salt in their environment, but tend to grow better without it.

Bacteria across many different phyla are halophilic, or halotolerant. The salty oceans harbor an enormous biomass of cyanobacteria, gram-negative and -positive bacteria can be found in salty soils, and many different phyla of bacteria are found in hypersaline lakes. In the domain of archaea, we find many extremely halophilic species. They are the most abundant organisms at the highest water salinities, i.e. greater than 20-25% NaCl (w/w) (Oren, 2002). Lastly, unicellular eukaryotes, like fungi, protists, and algae can also be found in some of the earth’s most saline environments. There are also a few halophilic multicellular organisms, e.g. insects, invertebrates, and some plants.

Fresh water habitats, like bogs, lakes, and rivers, often contain less than 0.05 % (w/w) dissolved salts, and thus do not harbor halophiles. The worlds’ oceans have an average salt content of 3.5% (w/w), of which 86 % are comprised of Na- or Cl-ions (Dickson, 2005). Water with salt contents higher than in the oceans can be found occurring naturally throughout the planet, e.g. extremely salty lakes, such as the Dead Sea (Jordan/Israel) and Great Salt Lake (USA). They form when salt rich water flowing into the lake cannot leave because the lake has no outlets. When the water evaporates, dissolved salts are concentrated to the saturation point, which is ~30 % (w/w) for NaCl. Even in lakes approaching this salinity, dense microbial communities can be found (Oren, 2002).
1.1.2 Salt survival strategies

Halophilic and halotolerant microorganisms use one of two major strategies for retaining sufficient water in their cytosol at hypersaline environments (DasSarma and DasSarma, 2001). The first is called the “salt in strategy”, and involves accumulation of high concentrations of KCl intracellularly. This enables the organism to balance their cytoplasm osmotically with their surrounding media. Organisms which employ this strategy have evolved acidic proteins to be able to function in a high salt environment. Such organisms generally cannot survive low salt media. This “salt-in” strategy is primarily used by aerobic, extremely halophilic archaea and some anaerobic bacteria (Oren, 2008). The “salt-in” strategy has the disadvantage that organisms cannot adapt to variations in salinity; however an advantage is that this way of balancing osmolarity is energetically relatively cheap.

The second strategy, “salt out”, is to exclude salts from their cytosol, and instead accumulate small organic molecules, known as compatible solutes, osmoprotectants, or osmolytes. These molecules balance out the osmotic extracellular and intracellular difference, without interfering with protein enzymatic function, or other biomolecules. Most halophilic bacteria use compatible solutes for osmoadaptation (Oren, 2008), as can be seen in figure 1.1. The “salt-out” strategy has the advantage that organisms are adaptable to variations in salinity; however the disadvantage is that the enzymatic synthesis of compatible solutes is energetically expensive.

Figure 1.1 Distribution of halophilic microorganisms within the tree of life. The tree is based on small subunit rRNA gene sequences. Phyla with colored boxes contain at least one halophilic representative (e.g. the Bacteroidetes, of which Salinibacter ruber is the sole halophilic member described to date). Blue boxes represent phyla that utilize the “salt in” strategy, orange boxes represents the “salt out” strategy, and the Firmicutes has species representatives with either strategy. Adapted from (Oren, 2008).
1.1.3 Compatible solutes

Compatible solutes are small and highly soluble molecules, comprising of only a limited number of compounds (Empadinhas and da Costa, 2008). They can be divided into two major groups: 1) Sugars and polyols (e.g. glycerol), and 2) alpha- and beta-amino acids and their derivatives. These compounds are called compatible solutes because in addition to serve as an osmolyte they are also compatible with the cells’ biomolecules, even when accumulated at high concentrations. Many microorganisms synthesize compatible solutes *de novo*; however, they preferably transport them actively across the cell membrane whenever the osmoprotectant can be found in the environment. Other microorganisms rely exclusively on uptake of compatible solutes from their surroundings. When faced with a decrease in external salinity levels, some microorganisms are able to rapidly extrude their accumulated organic solutes in order to achieve osmotic balance.

In addition to the osmotically beneficial effect of compatible solutes, they are also able to stabilize biomolecules under stress conditions, e.g. salt stress, desiccation, freezing, or elevated temperatures. Their effect on protein stability is likely explained by the “preferential exclusion model” (Arakawa and Timasheff, 1985). The model states that the compatible solute is preferentially excluded from the immediate protein surface, thereby forcing the protein to stay in its native conformation.

1.1.4 Metabolic engineering of crop plants with compatible solutes

Salinity is a major limiting factor for crop productivity and quality. Much of the world’s arable land is affected by relatively high salinity. Although variable, crop species generally do not tolerate water with more than 1% salt (Flowers, 2004). Consequently, salt tolerant crops have been sought after by numerous conventional breeding and genetic engineering experiments.

Although salt tolerant plants have been created, the results of these efforts have shown that there are several limitations to the methods and strategies currently used. As salt tolerance is a complex genetically and physiologically trait, it is understandably hard to identify single or few genes that confer a radical increase in salt tolerance (Yu *et al.*, 2012). Furthermore, large scale tests in farm fields are lacking, but are required in order to give a true indication of any increased salt tolerance.

Genes responsible for production of compatible solutes have been metabolically engineered into various plants (Rontein *et al.*, 2002). Although there are some positive results from such experiments, a number of problems seem to hinder the development of genuine salt resistance. First, while there is some work on major crops, most has been done with the model plants Arabidopsis and tobacco.
Second, the engineered levels of compatible solutes are generally low and the increases in tolerance small. Third, the genes used often come from bacteria, and thus poor translation of the mRNA may be a problem, due to divergent codon preferences in bacteria and plants. Fourth, low availability of substrates for production of compatible solutes might be a problem. Substrate exhaustion might even confer some side effects on the plant, such as inhibition of growth. Substrates may also be constrained to specific organelles or compartments. Little is known regarding the latter problem, hindering the improvement of salt tolerance in plants.

1.2 Ectoine

1.2.1 Discovery and characterization

During investigations on the extremely halophilic phototrophic bacterium *Ectothiorhodospira halochlori* researchers discovered a novel compatible solute: the small amino acid derivative 1,4,5,6-Tetrahydro-2-methyl-4-pyrimidinecarboxylic acid (Galinski *et al.*, 1985). As there were no previous references to this molecule, the trivial name “ectoine” was given. The following years ectoine was found in many other halophilic bacteria, and it is now reported to be the most abundant compatible solute of aerobic heterotrophic eubacteria (Kolp *et al.*, 2006).

The proteins necessary for the biosynthesis of ectoine were found to be encoded by three genes, named *ectA*, *ectB*, and *ectC*, organized as an operon and transcribed from a single promoter (Louis and Galinski, 1997). A subset of ectoine-producing bacteria has an additional gene, *ectD*, which codes for an enzyme that hydroxylates ectoine to 5-hydroxyectoine, which also serves as a compatible solute in some bacteria. Each of the *ectABCD* genes codes for an enzyme that catalyzes a step in the conversion of L-aspartate-β-semialdehyde (an aspartate precursor) to ectoine/hydroxyectoine (figure 1.2).
1.2.2 Commercial interest in ectoine

A German company, Bitop AG (http://www.bitop.de), is today the only large scale commercial producer of ectoine. The company was founded in 1993 to explore the economic potential of extremophile microorganisms, and in particular ectoine production. Since 2001 they have sold ectoine for use in cosmetic products, and in 2007 the first ectoine containing medical product, a nasal spray, was launched in Germany. Moreover, ectoine is also sold and licensed as a biomolecule stabilizer for use in molecular biology applications, since ectoine protects biomolecules from stresses (thermostability, degradation, oxidation, protein stabilization, DNA stabilization, osmotic stress, detergents) (Pastor et al., 2010).

Ectoine is produced in tons annually by Bitop AG, using a fermentation technique called bacterial milking (Melmer and Schwarz, 2009). In this system the natural ectoine/hydroxyectoine-producing halophilic bacterium *Halomonas elongata* is grown in a hyperosmotic medium at 15% NaCl, and subsequently given a hypoosmotic downshock to 3% NaCl. This triggers the bacteria to release compatible solutes into the medium which are then isolated and purified. The downshocked bacteria are then fed into the growing chamber at 15% NaCl again.

Improvements on the bacterial milking technique are actively researched. The current method is a cumbersome multi-step process, and the producer strain creates a mix of different compatible solutes, from which ectoine has to be purified. Genetic engineering experiments, and new wild-type strains, can potentially increase the yield and quality of ectoine, and/or hydroxyectoine.
1.2.3 Ectoine in metabolic engineering

The *ectABC* genes have been successfully integrated into the nuclear genome of both tobacco, and tomato plants, using *Agrobacterium tumefaciens* mediated transformation (Moghaieb *et al.*, 2006; Moghaieb *et al.*, 2011). The genes were transcribed from endogenous plant promoters. The plants accumulated ectoine, and showed increased resistance towards salinity compared to wild types. The experiments provide valuable insights into the mechanisms responsible for plant growth inhibition by salt, and effectiveness of ectoine as an engineered compatible solute.

1.3 The model organism *Chlamydomonas reinhardtii*

1.3.1 General features

*Chlamydomonas reinhardtii* (hereafter referred to as *Chlamydomonas*) is a eukaryotic, unicellular, photosynthetic, green alga that was isolated from soil habitats in North America, in the 1940s and 1950s. Since then it has been developed as a laboratory organism, and emerged as one of the preferred model systems for diverse areas of cell and molecular biology (Harris, 2001). The alga has proven especially useful in understanding chloroplast photosynthesis and the eukaryotic flagellum, as well as other areas such as centrioles, light perception, cell-cell recognition and cell cycle control.

The basic morphological features of *Chlamydomonas* are a cell of oval shape at approximately 10 µm in diameter with two anterior flagella of 10 to 12 µm in length. Two thirds of its cytoplasm is occupied by a single chloroplast, and the rest of the cytosol harbors the nucleus, endoplasmic reticulum, Golgi apparatus, mitochondria, contractile vacuoles, and other structures. It has a cell wall composed of seven layers of glycoproteins.

*Chlamydomonas* owes its usefulness as a model organism to many of its attributes. Its ability to grow heterotrophically in the absence of light, provided acetate, or another reduced carbon source is supplied in the medium, is particularly useful for photosynthesis research. This is a feature land plants do not have, and makes it possible to study mutants of photosynthetic function. Other desirable features are that of sexual mating, short generation time (~6 hours), growth on agar plates, and more recently well-established genetic transformation procedures for nuclear, chloroplast and mitochondrial genomes. The genome of *Chlamydomonas* has been fully sequenced. The linear mitochondrial genome (15.8 kb) was fully sequenced in 1993 (Vahrenholz *et al.*, 1993), the circular chloroplast genome (203.4 kb) in 2002 (Maul *et al.*, 2002), and the 17 linear chromosomes of the nucleus (121 Mb) were sequenced in 2007 (Merchant *et al.*, 2007).
Chlamydomonas is easily grown in liquid or agar media, and has no requirements for supplementary vitamins or other co-factors. Optimal growth temperature is from 20 ° to 36 ° Celsius, and it grows fastest phototrophically under continuous light and extra CO₂ bubbling.

1.3.2 The Chlamydomonas chloroplast as a protein expression system

Research into the Chlamydomonas chloroplast as a recombinant protein expression factory has been intense in recent years (Potvin and Zhang, 2010). A recombinant alga can have a large fraction of its total protein be recombinant, it can grow phototrophically, and grow in a closed system reducing the risk of environmental release. This makes it a potentially superior expression system.

However, for high levels of protein production several aspects, that are still being elucidated, have to be considered. Researchers report that altering transgene codons to better suit Chlamydomonas chloroplast codon usage is the single most important factor determining successful transgene expression (Potvin and Zhang, 2010). Another interesting experiment showed that recombinant protein yields varied from 0.88% to 20.9% of total cell protein in algal transformants, which had all been transformed with the same site-specific (homologous) transformation vector (Surzycki et al., 2009). This was speculated to be due to undetected integration events in the chloroplast, nucleus, or mitochondrion that might negatively or positively affect protein expression. Accordingly, Surzycki recommended screening multiple transformants for high expression, and warned that previous conclusions on expression yields might have been influenced by the random variability in yields.

Focus has also revolved around finding optimal 5’- and 3’-regions flanking the transgene coding sequence for high transcription rates, insertion of endogenous Chlamydomonas introns, and fusion constructs of endogenous proteins with transgenes (Potvin and Zhang, 2010). Proteolytic degradation in the chloroplast has been reported to be limited and thus may not be a major concern for chloroplast protein expression (Potvin and Zhang, 2010). However some reports indicate that protease activity needs to be suppressed for maximum yields (Surzycki et al., 2009)

1.3.3 Codon bias in the Chlamydomonas chloroplast

A codon is a three-letter nucleotide sequence that represents either a particular amino acid, start of translation, or stop of translation, for living cells’ protein synthesis machinery. All amino acids can be represented by two or more codon varieties, except for methionine (also serves as start signal) and tryptophan, which only have one codon each. Different organisms vary in what codons are used most for a particular amino acid, a phenomenon called codon bias (Ermolaeva, 2001).
Different explanations are offered for the observed codon bias, however it has been shown experimentally that mRNA with preferred codons are translated faster than artificially modified mRNA that contains rare codons (Ermolaeva, 2001). Moreover, highly expressed genes exhibit a greater codon bias than more infrequently expressed genes. Codon bias is correlated with the relative abundance of tRNA molecules and genes in a specific organism, probably explaining the mentioned observations.

The *Chlamydomonas* chloroplast genome, which has its own translation machinery, exhibits a high degree of codon bias (Nakamura *et al.*, 2000) (table 1.). Consequently, researchers that have expressed recombinant genes in the *Chlamydomonas* chloroplast have detected significantly more protein when codons were altered to match that of highly expressed chloroplast genes (Franklin *et al.*, 2002; Surzycki *et al.*, 2009).

### Table 1 Codon usage in the *C. reinhardtii* chloroplast (Nakamura *et al.*, 2000). Fields displayed for each codon from left to right are: codon triplet, one letter amino acid abbreviation, fraction for amino acid, frequency per thousand triplets, and number of instances in genome.

<table>
<thead>
<tr>
<th>codon triplet</th>
<th><em>Chlamydomonas</em></th>
<th>frequency (per thousand)</th>
<th>number</th>
</tr>
</thead>
<tbody>
<tr>
<td>UUU T 0.66 33.4</td>
<td>0.66 33.4</td>
<td>0.66 33.4</td>
<td></td>
</tr>
<tr>
<td>UUC T 0.34 17.1</td>
<td>0.34 17.1</td>
<td>0.34 17.1</td>
<td></td>
</tr>
<tr>
<td>UUA L 0.72 77.7</td>
<td>0.72 77.7</td>
<td>0.72 77.7</td>
<td></td>
</tr>
<tr>
<td>UUG L 0.04 4.3</td>
<td>0.04 4.3</td>
<td>0.04 4.3</td>
<td></td>
</tr>
<tr>
<td>CUU U 0.13 14.3</td>
<td>0.13 14.3</td>
<td>0.13 14.3</td>
<td></td>
</tr>
<tr>
<td>CUC U 0.01 1.0</td>
<td>0.01 1.0</td>
<td>0.01 1.0</td>
<td></td>
</tr>
<tr>
<td>CUA A 0.06 6.6</td>
<td>0.06 6.6</td>
<td>0.06 6.6</td>
<td></td>
</tr>
<tr>
<td>CGU G 0.03 3.7</td>
<td>0.03 3.7</td>
<td>0.03 3.7</td>
<td></td>
</tr>
<tr>
<td>AUU I 0.77 51.4</td>
<td>0.77 51.4</td>
<td>0.77 51.4</td>
<td></td>
</tr>
<tr>
<td>AUG U 1.00 22.5</td>
<td>1.00 22.5</td>
<td>1.00 22.5</td>
<td></td>
</tr>
<tr>
<td>GUU V 0.46 29.3</td>
<td>0.46 29.3</td>
<td>0.46 29.3</td>
<td></td>
</tr>
<tr>
<td>GUA V 0.41 26.0</td>
<td>0.41 26.0</td>
<td>0.41 26.0</td>
<td></td>
</tr>
<tr>
<td>GUG V 0.09 5.6</td>
<td>0.09 5.6</td>
<td>0.09 5.6</td>
<td></td>
</tr>
</tbody>
</table>

1.3.4 *Chlamydomonas* chloroplast transformation

Genetic modification of the *Chlamydomonas* chloroplast genome *in vivo* is now a routine task thanks to developments of the past. Today, researchers can do gene knockouts, specific gene mutagenesis, and foreign gene expression (Purton, 2007). An important mechanism allowing for all of these mutational strategies is the fact that the chloroplast has a working homologous recombination system. The circular chloroplast chromosome of *Chlamydomonas* is present in 50-80 copies, and thus
homologous recombination occurs routinely, possibly as a part of maintenance and repair procedures.

When a foreign DNA sequence, with a few hundred base pairs of sequence homology to the chloroplast chromosome, is artificially introduced to the chloroplast, it will integrate by homologous recombination (Blowers et al., 1989). Furthermore; if some foreign gene, or other DNA sequence, is flanked by chloroplast homologous sequences, the whole chimeric construct will integrate stably into the genome. This can be exploited to do gene knockout, gene engineering, and foreign gene expression. The actual mechanism of DNA integration is not known, but is best envisaged as a simple double crossover event as shown in figure 1.3.

![Figure 1.3. Integration of a foreign gene (gene X) to the Chlamydomonas chloroplast circular genome (ptDNA). A and B denote homologous sequences present on both the transformation vector and endogenous plastid DNA. Figure adapted from (Bock, 2007).](image)

Initially an introduced transgene will generally only transform a fraction of the 50-80 chromosome copies in the chloroplast; the chloroplast will be heteroplasmonic. However; if a positive selectable marker is part of the transformation construct, the transformant can be grown under selectable conditions until homoplasmicity is achieved. This generally requires iterative screening and selection.

1.3.5 Transformation by microprojectile bombardment

Transformation by microprojectile bombardment is a highly efficient method of delivering foreign DNA to any type of cell. In essence, it is a system where DNA is adhered to smaller-than-cell sized gold or tungsten particles, and subsequently shot with high velocity onto biological material. The particles will have enough kinetic energy to penetrate the cell wall, and cell membranes, and deposit the DNA randomly in nucleus, organelles, or cytosol. This delivery system often yields a higher number of transformants than other methods, and can be used to transform most kinds of cell. It can be used for transforming DNA by homologous recombination, or random integration (non-homologous integration).
Today, commercial suppliers offer an integrated system which accelerates heavy metal particles with high pressure helium gas. This allows a clean and sterile delivery system. The biolistic system is used extensively for introducing exogenous DNA into the *Chlamydomonas* chloroplasts.

1.3.6 Chloroplast transformation with photosynthesis selection marker

The first successful integration of a foreign gene into the *Chlamydomonas* chloroplast genome was done in 1989 (Blowers *et al.*, 1989). They transformed a *Chlamydomonas* mutant that was known to have a 2.5 kilobase pair (kb) deletion in the chloroplast genome. The deletion spans across a substantial part of the chloroplast ATP synthase subunit beta (*atpB*) gene, rendering this mutant incapable of photosynthetic growth. However, it had previously been shown that photosynthesis could be restored by bombarding the cells with a 5.3 kb sequence from wild type *Chlamydomonas* that contained the 2.5 kb deletion sequence. Thus the *atpB* gene could integrate with the homologous flanking sequences, restoring photosynthesis. Selection of positive transformants was then done by growing bombarded algae in light on medium without a carbon source. Under this condition only the alga that had restored the *atpB* gene would be able to grow.

By cloning the bacterial β-glucuronidase (GUS) gene (fused to a maize chloroplast promoter), inserting it into a KpnI site in the 5.3 kb wild type sequence, and bombarding this DNA onto the photosynthesis mutant, Blowers *et al.* successfully transformed the *Chlamydomonas* chloroplast with a foreign gene (figure 1.4). They were able to detect transcribed mRNA from the bacterial gene; however, no attempts were made to measure the presence of the gene protein product at that time. This transformation method had the advantage of using a selection system that restores the wild type genotype in *Chlamydomonas*, thus eliminating the need for an exogenous selection marker.
Figure 1.4 Schematic overview of the DNA sequences involved in the first successful integration of a foreign gene into the *Chlamydomonas* chloroplast genome. (A) shows the 5.3 kb BamHI-EcoRI fragment isolated from wild type *Chlamydomonas* chloroplast DNA containing the full *atpB* gene, that was used to restore photosynthetic growth in *atpB* deletion mutants. (B) shows the same DNA fragment as in (A) but with a transgene inserted in a KpnI site. The arrows between the “transformation DNA” and “deletion mutant DNA” indicate the homologous recombination event that leads to integration of the transformation DNA into the deletion mutant DNA. Below the “deletion mutant DNA” is indicated the 2.5 kb deletion, and a measurement scale. Based on (Blowers *et al.*, 1989).

1.4 Ectoine production in *Chlamydomonas* by expression of *ectA* in the chloroplast

The chloroplast is the primary site of amino acid synthesis in *Chlamydomonas*, and would accordingly be a prime site for the metabolically engineered expression of ectoine.

The chloroplast harbours the substrate for EctB, the enzyme that catalyzes the initial step of ectoine biosynthesis. The reaction is L-aspartate-β-semialdehyde -> L-2,4-diaminobutyrate, and is a transaminase reaction with glutamate as substrate (figure 1.2). Transaminase-reactions are common in amino acid metabolism, and relatively similar reactions to EctB are being catalyzed by a variety of enzymes in the chloroplast. It is possible that some of them can act on L-aspartate-β-semialdehyde, and thus catalyze the same reaction as EctB. This would render EctB redundant in a transgenic *Chlamydomonas* ectoine producer (Dr. Galinski, University of Bonn (Germany), personal communication).
EctA catalyzes the next step (L-2,4-diaminobutyrate -> Nγ-acetyl-L-2,4-diaminobutyrate) of ectoine biosynthesis, with acetyl-CoA as a cofactor, which is abundant in the chloroplast. The terminal step (Nγ-acetyl-L-2,4-diaminobutyrate -> ectoine) is a dehydratase reaction that also circularize the molecule, and is catalyzed by EctC. This step might also be catalyzed by other enzymes of the chloroplast, or might occur spontaneously.

If the activities of EctB and EctC are redundant in the *Chlamydomonas* chloroplast, it would be sufficient to express EctA in order to synthesize ectoine. This would greatly simplify the process of creating an ectoine producing *Chlamydomonas* strain, as only one gene would have to be cloned with appropriate promoters and terminator sequences.

The potential application of engineered synthesis of ectoine in *Chlamydomonas* is two-pronged. First, the prospect of increased salt tolerance by metabolic engineering of the chloroplast can be investigated by using *Chlamydomonas* as a model for higher plants. The potential benefits of chloroplast ectoine expression include high levels of substrates, and high expression yields of transgene protein.

Second, an ectoine-producing *Chlamydomonas* strain has the potential to replace current industrial producers of ectoine. *Chlamydomonas* has several characteristics that could make it a superior ectoine producer, e.g. simplification of culturing procedure, phototrophic growth, and no endogenous production of contaminating compatible solutes.
Aim of project

The main goals of this project were:

1. To create a transgenic *Chlamydomonas reinhardtii* cell line that harbors a functional *ectA* gene in the chloroplast.
2. Evaluate the expression of the *ectA* gene, and expression of ectoine.

This can be divided into the following sub-goals:

1. To create a plasmid construct that can be used for transformation of the chloroplast genome with *ectA*. This construct would have to contain promoter, and terminator regions for the transcription of *ectA*, in addition to flanking 5’ and 3’ untranslated regions (UTR) that enables stabilization and translation of the transcript. Moreover, the construct would have to contain a selection marker, and sequences that are homologous to chloroplast DNA, for the stable homologous integration of the construct.
2. To transform *Chlamydomonas* with the generated plasmid construct, by microprojectile bombardment, and selection of positive transformants.
3. To analyze any positive transformants at the DNA, RNA, protein, and ectoine level.
4. To create a codon optimized version of the *ectA* gene, and create a transformation plasmid construct with the gene in the same way as for *ectA*. Likewise, transformants would be generated, selected, and analyzed.
2.0 Materials and Methods

Recipes for solutions and mixtures used can be found in the Appendix 4.

2.1 Work with Escherichia coli (E. coli)

Chemically competent E. coli TB1 or dam-/dcm- cells (NEB) were used for all cloning steps.

2.1.1 Preparation of agar growth plates with ampicillin

Ampicillin is sensitive to temperature degradation, so ampicillin is added only to cooled LB, and the final plates are stored in a refrigerator to extend their shelf life.

Procedure for 1 L (~40 plates):
- 1 L of lysogeny broth (LB) with 1.5 % agar is prepared and autoclaved.
- The solution is cooled to 50 °C, and 1 mL of ampicillin [60 mg/ml] is added, to a final concentration of 60 µg/ml.
- The solution is poured into sterile plastic plates under a sterile hood, and allowed to solidify.
- After solidification the plates are stacked, turned upside down (to avoid water condensation), sealed in plastic bags, and stored at 4 °C.

2.1.2 Preparation of competent E. coli TB1 cells (CaCl₂ method)

E. coli is normally prepared for competency in large batches, and stored at -80 °C. When needed, the cells are taken out, mixed with DNA and given a heat shock. Competent dam-/dcm- cells were used as supplied.

Procedure:
-Use sterile pipette tips and equipment
-Grow E. coli cells (3 ml strain TB1) at 37 °C on LB medium in a tube overnight on a rotating wheel.
-Inoculate a 100 mL culture (LB medium) in a 250 ml Erlenmeyer flask with 2 mL of the tube, and grow on a shaker at 37 °C.
-Occasionally check absorbance of the culture at 600 nm in a 1 cm disposable plastic cuvette (use LB medium as blank).
-When the optical density (OD) is around 0.6 (should be around 2.5 hours after starting the culture) harvest the cells in two sterile 50 mL capped plastic tubes (5000 rpm (SS34 rotor), 10 minutes, 4 °C.
-Discard supernatant and resuspend the pellet in each tube in 20 mL ice-cold sterile 0.1 M CaCl₂. Leave tubes on ice for 10 minutes.
-Spin tubes as before, discard supernatant, and resuspend each pellet in ice-cold sterile 2 mL 0.1 M CaCl₂ with 15 % glycerol.
Dispense cells in 50 µL aliquots into sterile 1.5 mL microfuge tubes on ice and freeze immediately in liquid nitrogen, or in -80 ° C freezer. Cells remain competent for several months. Competency may be checked by transformation with a control plasmid, like pUC or pBluescrips, at a concentration of 1 ng/µL.

2.1.3 Transformation of competent E. coli

Procedure:
- Melt an aliquot (50 µL) of frozen competent cells on ice, and add 3 µL of pure DNA (1 ng/µL) or 3 µL of a ligation reaction. Mix and leave tube on ice for 30 minutes.
- Prepare a water bath of 42 ° C, and incubate the tube in the bath for 1 minute. Immediately cool down on ice afterwards.
- Add 0.8 mL of sterile LB medium at room temperature and transfer the mix to a 15 mL plastic tube. Incubate on a rotating wheel at 37 ° C for 45 – 60 minutes.
- Plate the suspension as described in chapter 2.1.4.

2.1.4 Growth of E. coli on agar plates with ampicillin

Procedure:
- Everything, except the overnight incubation, should be done in a sterile hood.
- A glass rod is sterilized by burning with alcohol and cooled down in air.
- 75 µL of E. coli culture is pipetted onto a plate (if too many colonies appear, use diluted culture).
- The plate is put on a hand-operated rotating device.
- The plate is rotated while the glass rod is used for spreading the E. coli culture evenly around the plate.
- The plate is left for about 3 minutes to allow the liquid to absorb into the agar.
- Put the lid on the plate, turn the plate upside-down, and incubate at 37 ° C for 16 – 24 hours.
- Use immediately, or store at 4 ° C. Can be stored in the cold for several weeks.

2.1.5 Culturing of E. coli agar plate colonies

Procedure:
- 3 mL of lysogeny broth (LB) with ampicillin [60µg/ml] is added to a sterile 15 mL tube with loose caps for air ventilation.
- A single colony is picked from a plate, with a sterile pipette tip.
- The pipette tip is put inside the tube, making sure the tip with the colony is in contact with the LB medium.
- The tube is incubated overnight at 37 ° C on a rotating wheel.
- The next day, the tube can be used for plasmid miniprep isolation (chapter 2.1.7)
2.1.6 Storage of *E. coli* at -80 °C

Procedure:
- Take 0.7 mL of the *E. coli* culture and mix with 0.3 mL of 50% glycerol in a 2 mL tube with screw cap.
- Store in -80 °C freezer. Cells are viable for several years.

2.1.7 Miniprep plasmid isolation from *E. coli*

Procedure:
- Transfer 1.5 mL of an *E. coli* culture into a 1.5 mL microfuge tube.
- Centrifuge at 13,000 g for about 30 seconds.
- Discard supernatant, and resuspend the bacterial pellet in 100 µL ice-cold TEG buffer.
- Leave for 5 minutes at room temperature.
- Add 200 µL of NaOH/SDS [0.2 N, 1% (w/w)] solution (prepare fresh). Mix by inversion, and incubate on ice for 5 minutes.
- Add 150 µL ice-cold potassium acetate [5 M K, 3 M acetate]. Mix by inversion, and incubate on ice for 5 minutes.
- Centrifuge at 13,000 g at 4 °C for 5 minutes.
- Centrifuge at 13,000 g for 2 minutes.
- Centrifuge at 13,000 g for 2 minutes.
- Transfer 310 µL of upper phase to a new tube. Add 750 µL of ice-cold 96% ethanol. Mix by vortexing, and leave on ice for 10 minutes.
- Centrifuge at 13,000 g at 4 °C for 10 minutes.
- Discard supernatant, and add 1 mL of ethanol [70%] to the pellet. Mix by inversion.
- Centrifuge at 13,000 g at 4 °C for 5 minutes.
- Discard supernatant, and dry the pellet by leaving at room temperature for 10 minutes, or dry in a vacuum centrifuge.
- Dissolve the pellet in 15 µL of dH2O.

2.1.8 Maxiprep plasmid isolation from *E. coli*

- Inoculate 5 µL or 1 colony from a plate of *E. coli* culture in 100 mL LB with ampicillin [60 µg/mL], and grow the culture overnight at 37 °C on a shaker.
- Centrifuge at 6,000 g at 4 °C for 5 minutes.
- Discard supernatant, and resuspend cells in 3.6 mL ice-cold TEG buffer.
- Add 0.4 mL of lysozyme [10 mg/ml] in TEG (prepare fresh). Leave at room
temperature for 5 minutes, and then leave on ice for 5 minutes.
-Add 8 mL NaOH/SDS [0.2 N, 1 % (w/w)] solution (prepare fresh). Mix by inversion, and leave on ice for 5 minutes.
-Add 6 mL ice-cold potassium acetate [5 M K, 3 M acetate]. Mix by inversion, and incubate on ice for 5 minutes.
-Centrifuge at 6,000 g at 4 °C for 10 minutes.
-Transfer supernatant to a new tube by filtering through a cheesecloth. Add 12.5 mL isopropanol. Mix by vortexing. Leave at room temperature for 15 minutes.
-Centrifuge at 6,000 g at room temperature for 10 minutes.
-Discard supernatant, and leave tube for drying in room temperature for 10 minutes.
-Determine the weight of the solution and add TE [50 mM Tris (pH 8), 1 mM EDTA] buffer to 4.2 grams.
-Add 4.5 g CsCl, mix until dissolved, and warm solution to room temperature.
-Add 0.5 mL ethidium bromide [10 mg/ml], and mix by pipetting.
-Centrifuge at 6,000 g at room temperature for 5 minutes.
-Transfer supernatant to Beckman OptiSeal tubes, and load tube into VTi 65.2 rotor.
-Centrifuge at 50,000 g at 15 °C for 15 hours or more. Decelerate rotor without brake.
-Carefully transfer the ultracentrifugation tube to a clamp, and illuminate the tube with 350 nm UV-light. Extract the middle band with a 2 mL syringe. Volume should be around 0.5 mL.
-Remove ethidium bromide by extracting with 0.75 mL isopropanol/water [7:1 v/v] up to five times.
-Dialyze against sterile 200 mL TE buffer at 4 °C for 1 hour. Replace with fresh buffer every hour twice or three times.
-Transfer DNA solution to a tube with screw cap, and store in –20 °C freezer.
-Determine the concentration by measuring the absorption at 260 nm (chapter 2.2.4 (dilution 1:100))

2.2 Work with DNA

2.2.1 Agarose gel electrophoresis

Procedure (1 % agarose gel):
-Measure 60 mL TAE [1 X] buffer in an Erlenmeyer flask.
-Add 0.6 g agarose. Cover Erlenmeyer flask with plastic foil, and bring to a boil in a microwave.
-Cool down flask to 50 °C and add 10 μL of ethidium bromide [1 mg/mL].
-Pour solution into a tray, and insert comb.
-Wait up to one hour for solidification, and immerse the gel in electrophoresis chamber. Pour TAE [1 X] buffer into the chamber so that it just submerges the gel.
-Mix 9 μL of DNA sample with 1 μL of agarose gel loading buffer. Load 10 μL in each well. Add a DNA standard in one of the wells.
-Apply a voltage according to electrophoresis chamber specifications. Usually 90
volts.  
- After adequate electrophoresis, visualize the DNA bands under UV-light.

2.2.2 Purification of DNA fragments from agarose gel

Specific DNA fragments were isolated from agarose gels using the Illustra™ purification kit from GE Healthcare.

2.2.3 Quantification of DNA (dot spot)

Procedure:
- Prepare a standard of DNA in water solutions, with increasing concentrations [0 ng/µL, 2.5 ng/µL, 5 ng/µL, 7.5 ng/µL, 10 ng/µL, 15 ng/µL, and 20 ng/µL].
- Place a series of 2 µL drops of ethidium bromide-water solution [2 µg/mL] on a plastic petri dish that is transparent to UV-light. Place 7 drops for the DNA-standards, and additional drops according to the number of samples you wish to measure.
- Add 2 µL of the DNA-standards to each ethidium bromide drop, mixing by pipetting within the drop a few times. Usually up to a 100-fold dilution is required to lower the sample DNA-concentration to match that of the standards range.
- Add 2 µL of the sample DNA to a ethidium bromide drop, mix by pipetting.
- Compare the sample fluorescence under UV-light to that of the standards, and estimate DNA concentration.

2.2.4 Quantification of DNA by ultraviolet light absorption

Procedure:
- Calibrate/zero the absorption apparatus at 260 nm with a control sample, using the liquid that was used to resuspend the DNA with.
- Dilute sample as needed, and measure optic density (OD).
- OD 1.0 = 50 µg DNA/ml (40 µg RNA/ml).

2.3 Subcloning

2.3.1 Plasmids

The ect_operon_stutzeri plasmid was provided by Dr. Galinski (University of Bonn, Germany), and contains the cloned ectABCD operon from *Pseudomonas stutzeri* (Seip et al., 2011). The plasmid SK+ 157_Eco47 is described in (Salvador et al., 2011) (the plasmid SK+157, with the Eco47 modification). The pCrc_32 plasmid is described in (Blowers et al., 1993), where it is named pCrc32. The geneart_OectA plasmid was synthesized by Life Technologies™ GeneArt® service.
Prior to subcloning of *ectA* and *OectA*, SK+\_157\_Eco47 (which contains the *rbcL* 5’ region) was modified to have a NdeI site, instead of an Eco47 site. This was done by oligonucleotide cloning. Two complementary oligonucleotides (5'-oligo, and 3'-oligo) were annealed, phosphorylated, and cloned into the BspEI, and AfeI site of SK+\_157\_Eco47. The resulting plasmid, named SK+\_157\_NdeI, was transformed into competent *E. coli* TB1 cells, and plasmid was isolated by maxi preparation.

5’-oligo:
5’-
CCGGACAGATTATTTTAGGATCGTCAAAAGAAGTTACA
TTTATTTATACATAT
G-3’

3’ oligo:
5’-CATATGTATAAAATAATGTAACCTTCTTTTGACGATCCTAAAATAATCTGT-3’

![Figure 2.1 SK+\_157\_Eco47 plasmid with relevant restriction sites indicated.](image)

pBluescript is the vector backbone of this plasmid, derived from plasmid pBluescript SK+ (Stratagene, La Jolla, CA, USA).

2.3.2 Polymerase chain reaction (PCR)

PCR reactions was carried out using the DyNAzyme II DNA Polymerase (New England Biolabs (NEB)), with a Biometra thermocycler.

Primers used for cloning of *ectA* (restriction sites underlined):

pcr5’ :  5’-GTGCGCATATGCTACCCCTAAAA-3’ (NdeI site)
pcr3’ :  5’-GCACCTAGTGGCTACCCCTGGATTCTCCAG-3’ (SpeI site, NheI site)

Primers used for amplification of *atpB*:
5’primer: 5’-CAGAAGGTAAAATGCGTGGTATGG-3’
3’primer: 5’-GAATGGTTGAGATAAGACG-3’

Procedure (50 µL reaction):
- Add 32.5 µL sterile dH₂O to a PCR tube.
Add 5 µL 10x polymerase enzyme buffer.
Add 1.5 µL dNTPs [10 mM]
Add 5 µL plasmid template DNA [1 ng/µL]
Add 2.5 µL of sense-strand primer [10 pmol/µL] and 2.5 µL of anti-sense-strand primer [10 pmol/µL].
Add 1 µL of thermostable DNA polymerase [2 units/µL].
Mix with a pipette, centrifuge briefly to collect all liquid in bottom of tube, and insert tube into PCR thermal cycler.
The thermal cycler was set as follows:
Step 1: 98 ° C, 3 minutes
Step 2: 98 ° C, 15 seconds
Step 3: 54 ° C, 15 minutes
Step 4: 72 ° C, 45 seconds, repeat steps 2-4 30 times
Step 5: 72 ° C, 10 minutes

2.3.3 Restriction digestion of DNA

All restriction digestions were done with enzymes from NEB, following the protocols recommended by the supplier.

2.3.4 Partial restriction cutting of DNA

20 µg of SK+_ectA was partially digested with NheI by using 5 units of enzyme for 7 minutes at 37 ° C in a 50 µL reaction. After the reaction, the sample was mixed with loading buffer, and immediately loaded on an agarose gel and run, in order to stop the reaction.

2.3.5 Ligation

For all ligation reactions T4 DNA ligase (NEB) was used. The amount of vector used in a 10 µL reaction was 800 ng of a vector of 12 kb, and 200 ng of a vector of 3 kb and so on. The amount of insert used based on a molar ratio of 1.3:1 (insert:vector) for normal inserts (0.5 to 3 kb), and 5:1 for small inserts (less than 100 bp).

Procedure (10 µL reaction):
-Add your insert-DNA and vector-DNA (in H₂O) in a microfuge tube, not exceeding 6.5 µL.
-Add sterile dH₂O to raise total volume to 6.5 µL if needed.
-Incubate at 45 ° C for 5 minutes.
-Cool to room temperature.
-Add 1 µL T4 DNA ligase buffer (10x).
- Add 2 µL PEG 8000 [30 %, w/v].
- Add 0.5 µL T4 DNA ligase enzyme.
- Incubate at 19 °C for 3 hours.
- Use immediately.

2.3.6 Codon optimization of ectA

The codon optimized sequence of ectA (OectA) was generated manually using the codon frequency table generated from total Chlamydomonas chloroplast genes usage (table 1) (Nakamura et al., 2000). The original ectA sequence used was from Pseudomonas stutzeri (Seip et al., 2011). The actual sequence manipulation was carried out by Biruk Abrha (master student, Uwe Klein lab)

The sequence was generated as follows: Every codon that is listed (table 1) as being used less than 20 % for a specific amino acid, and was found to occur in the ectA sequence, was changed to the synonymous codon that is most used in Chlamydomonas chloroplast. E. g. when coding for leucine the CTC codon is only used 1 % of the time, whereas the most used codon TTA is used 72 % of the time. Accordingly, all CTC codons where changed to TTA in the OectA sequence.

Inadvertently, two codons were changed even though they did not fit the set criteria for optimization. This was due to a mix-up of two versions of the original sequence for ectA; one from an older sequencing project on a different strain of Pseudomonas stutzeri, rather than the sequence from the supplied ect_operon_stutzeri plasmid. This was discovered after sequencing the final vector constructs. However, the difference between the two mixed-up sequences were very small (less than 6 point mutations). All differences were silent mutations, affecting only the codon used, and thus did not make a significant difference in the optimization process. The inadvertent changes were: Asp-62 was changed from GAT (usage: 72%) to GAC (usage: 28%), and Phe-159 was changed from TTC (usage 34%) to TTT (usage: 66%).

2.3.7 Sequencing

Sequencing was done by Eurofins MWG/Operon (Germany). Primers for sequencing were custom designed to sequence the (O)ectA sequence, and parts of the flanking 5’- and 3’ regions.
2.4 Work with *Chlamydomonas*

The non-photosynthetic *atpB* deletion mutant strain ac-uc-221 (CC373), and wild type strain (CC125) of *Chlamydomonas* were originally obtained from the culture collection of the *Chlamydomonas* Genetics Center at University of Minnesota, MN, USA. The photosynthesis mutant is maintained in the dark in high salt high acetate (HSHA) media, and the wild type and transformants are maintained in the light in high salt (HS) media.

2.4.1 Preparation of solid media for *Chlamydomonas*

Procedure (1 L, enough for 40 plates):
- Prepare 1 L of high salt high acetate/high salt (HSHA/HS) media with 1.5 % agar. Autoclave for 20 minutes.
- The solution is cooled to 50 °C, and poured into sterile plastic plates under a sterile hood, and allowed to solidify.
- After solidification the plates are stacked, turned upside down (to avoid water condensation), sealed in plastic bags, and stored in room temperature.

2.4.2 Preparation of liquid medium for *Chlamydomonas*

Procedure (1 L):
- Prepare 1 L of HSHA/HS media in one large Erlenmeyer flask, or 250 mL flasks.
- Autoclave for 20 minutes.
- Store at room temperature.

2.4.3 Culturing the *Chlamydomonas* photosynthesis mutant for transformation.

Procedure:
- Inoculate a 100 mL solution of high salt high acetate (HSHA) medium with the photosynthesis mutant *Chlamydomonas*.
- Shield the culture from intense light by wrapping the flask in dark paper.
- Leave the culture on a shaker for 2 days.
- Inoculate ~5 mL of the culture to a new flask with 100 mL HSHA media.
- Leave the culture on a shaker for 2 days.
- Inoculate the whole culture to a new flask with 500 mL HSHA media.
- Keep the culture shielded from intense light.
- Leave the culture on a shaker for 1 day.
- The culture will be ready for transformation.
2.4.4 Harvesting and plating of the *Chlamydomonas* photosynthesis mutant for transformation

Procedure (6 plates):
- All steps should be done in low light.
- Centrifuge at 5,000 g at room temperature for 5 minutes 500 mL of the mutant culture in two sterile centrifuge bottles.
- Discard the supernatant and resuspend the cells in up to 500 µL high salt high acetate (HSHA) media.
- Heat sterilized soft agar [0.11 % agar in HSHA] in a microwave oven, and add 900 µL to two sterile microfuge tubes.
- Let tubes cool down to 42 °C on a heating block.
- When the soft agar has reached 42 °C add 100 µL of the resuspended *Chlamydomonas* to each microfuge tube, and mix gently.
- Transfer 300 µL of the cells to an HSHA agar plate (3 cm diameter). Spread the liquid as evenly as possible on the plates.
- Transfer 300 µL as above to more plates, until all cells have been used (should be 6 plates).
- Store the plates in the dark for 3 hours before microprojectile bombardment. Do not turn upside-down.

2.4.5 Microprojectile bombardment

Procedure:
- Transformation vector DNA is precipitated to gold particles according to instructions of the manufacturer of the particle delivery system (Biorad).
- The following steps are done in low light.
- The whole inside of the particle bombardment device is sterilized by wiping with ethyl alcohol.
- The particle bombardment device is assembled with the agar plates with photosynthesis mutant, and particle bombardment is carried out according to operating instructions.
- After bombardment, the plates are stored in the dark at room temperature for 1 day in order for the cells to recover. Do not turn upside-down.

2.4.6 Recovery and selection of transformants

Procedure:
- 1 day after microprojectile bombardment, the bombarded plates are transferred to a sterile hood.
- 400 µL of high salt (HS) media is added to each plate.
- A hand-held tool that has a rigid metal wire angled to 90 ° is used for scraping off
the thin layer of cells that are embedded in the soft agar. As many cells as possible are scraped into the HS medium that was added to the plate.
-All of the liquid on the plate is transferred to a fresh HS agar plate with a pipette, and spread out as evenly as possible using a glass rod.
-Repeat for each plate.
-The HS agar plates are put into light, but not turned upside-down.
-After 1 day, the plates are sealed with parafilm, and turned upside-down. They are kept under constant illumination.
-Colonies of transformed *Chlamydomonas* should appear after 2 weeks or more. These are picked in a sterile hood, and transferred to HS agar plates with a visual grid, and subsequently inoculated to liquid cultures.

2.4.7 Photosynthetic growth of *Chlamydomonas*

When growing *Chlamydomonas* photosynthetically the alga is cultured on solid or liquid media with no carbon-source other than CO$_2$, and with an adequate light-source. One, or preferably more fluorescent light-tubes are sufficient for supporting growth, at a distance of a few decimeter. The algae can grow under continuous light (24 hours a day).

2.4.8 Photosynthetic growth of *Chlamydomonas* with 2 % CO$_2$

*Chlamydomonas* is grown with an additional supply of CO$_2$ in liquid culture prior to DNA and RNA isolation.

Procedure:
-Under a sterile hood add 100 mL of high salt medium to a 250 mL glass tube that has gas inlet and outlet enabling bubbling by air. The tube should otherwise be sealed in order to prevent contamination.
-Inoculate the tube with a few mL of liquid *Chlamydomonas* culture.
-Place the tube in a water bath with a constant temperature of 32 °C.
-Connect the gas inlet to a source that supplies 2 % CO$_2$ in air. This can be achieved by bubbling CO$_2$ and air through water, at a rate of respectively 1 bubble per second and 20 bubbles per second, or a similar ratio, and leading the gases into the *Chlamydomonas* culture. The exact amount and rate of bubbling will only influence the speed of growth, and can be varied.
-After a few days the cultures should be dark green, and ready for harvest.
2.4.9 Total DNA isolation from *Chlamydomonas*

Procedure:
- Centrifuge at 5,000 g at room temperature for 5 minutes 40 mL of a *Chlamydomonas* culture containing ca. 2 million cells per mL.
- Discard supernatant and resuspend the pellet in 0.75 mL DNA extraction buffer [100 mM Tris pH 8.0, 50 mM Na$_2$-EDTA, 0.5 M NaCl, 10 mM β-mercaptoethanol], and transfer the suspension to a 2 mL microfuge tube.
- Add 60 µL SDS [21 % w/v], mix, and incubate for 15 minutes at 65 °C.
- Let cool down to room temperature and add 0.9 mL phenol (equilibrated with 0.1 M Tris pH 8.0). Mix carefully by inverting the tube.
- Centrifuge at 13,000 g at room temperature for 5 minutes.
- Transfer 750 µL of upper phase to a new 2 mL microfuge tube, add 750 µL phenol/chloroform/isoamylalcohol [25:24:1], and mix by inversion.
- Centrifuge at 13,000 g at room temperature for 5 minutes.
- Transfer 650 µL of upper phase to a new 1.5 mL microfuge tube, and add 650 µL of isopropanol. Mix by inversion and incubate at room temperature for 5 minutes, or until precipitate appears.
- Centrifuge at 4,000 g at room temperature for 2 minutes. Discard supernatant, and add 1 mL of ice-cold ethanol [70 %]. Mix by inversion.
- Centrifuge at 13,000 g at room temperature for 2 minutes. Discard supernatant, and dry pellet in a vacuum centrifuge, or by incubation at room temperature.
- Resuspend pellet in 90 µL TE buffer [10 mM tris pH 8.9, 1 mM Na$_2$-EDTA], add 10 µL RNase A [1 mg/ml], and incubate at 37 °C for 1 hour.
- Extract the mixture once with 100 µL phenol/chloroform/isoamylalcohol [25:24:1], and once with 100 µL chloroform/isoamylalcohol [24:1].
- Precipitate the DNA in freezer for up to 1 hour with ethanol (add Na-acetate to 0.3 M, then add 2 final volumes ethanol [96 %].
- Centrifuge at 13,000 g at 4 °C for 10 minutes. Discard supernatant, and add 1 mL of ice-cold ethanol [70 %]. Mix by inversion.
- Centrifuge at 13,000 g at 4 °C for 5 minutes. Discard supernatant, and dry pellet in a vacuum centrifuge, or by incubation at room temperature.
- Resuspend DNA in 20 µL of sterile dH$_2$O. Measure DNA concentration (chapter 2.2.3), and store in freezer or use immediately.
2.4.10 RNA isolation from *Chlamydomonas*

Only RNase-free consumables and solutions were used. Work quickly in the beginning. Keep samples always on ice.

Procedure:
- Centrifuge at 5,000 g at 4 °C for 5 minutes 40 mL of a *Chlamydomonas* culture containing ca. 2 million cells per mL.
- Discard the supernatant and resuspend the pellet in 1.5 mL ice-cold lysis buffer [0.6 M NaCl, 200 mM tris pH 8.0, 10 mM Na$_2$-EDTA].
- Add 150 µL RNase inhibitor [200 mM vanadyl ribonucleoside (NEB)].
- Transfer cells to a 15 mL tube containing 2 mL phenol (equilibrated with 0.1 M tris pH 8.0) and 1.5 mL SDS [4 %] preheated to 65 °C. Put a cap on the tube and mix.
- Incubate for 15 minutes at 65 °C. Mix occasionally, 3-4 times, by shaking.
- Cool down on ice and add 1 mL ice-cold chloroform/isoamylalcohol [24:1]. Mix.
- Centrifuge at 8,000 g at 4 °C for 5 minutes.
- Transfer 3 mL of the upper phase to another 15 mL tube containing 3 mL ice-cold phenol/chloroform/isoamylalcohol [25:24:1]. Mix.
- Centrifuge at 8,000 g at 4 °C for 5 minutes.
- Transfer 2.5 mL of the upper phase to another 15 mL tube containing 2.5 mL ice-cold phenol/chloroform/isoamylalcohol [25:24:1]. Mix.
- Centrifuge at 8,000 g at 4 °C for 5 minutes.
- Transfer 2 mL of the upper phase to another 15 mL tube containing 2.5 mL ice-cold isopropanol and 250 µL Na$_2$-acetate [3 M pH 5.2]. Mix and incubate at -20 °C for at least 1 hour.
- Centrifuge at 12,000 g at 4 °C for 15 minutes. Discard supernatant, and leave the tube inverted on a paper towel for 10 minutes in order to completely dry the pellet. Watch the pellet as it might be loose.
- Resuspend pellet in 300 µL DEPC-treated H$_2$O, and transfer to a sterile 1.5 mL microfuge tube containing 100 µL ice-cold LiCl [8 M]. Mix and incubate on ice for 2 hours.
- Centrifuge at 13,000 g at 4 °C for 30 minutes. Discard supernatant and resuspend pellet in 100 µL DEPC-treated H$_2$O. Keep on ice.
- Dilute 10 µL of the RNA solution in 1 mL DEPC-treated H$_2$O, and measure OD$_{260\text{nm}}$ (OD 1.0 = 40 µg RNA/mL). To the rest of the RNA solution add 10 µL Na-acetate [3 M pH 5.2] and 200 µL ice-cold ethanol [96 %]. Mix and incubate at -20 °C for 1 hour.
- Centrifuge at 13,000 g at 4 °C for 10 minutes. Discard supernatant and add 1 mL of ethanol [70 %]. Mix by inversion.
- Centrifuge at 13,000 g at 4 °C for 10 minutes. Discard supernatant and dry the pellet in a vacuum centrifuge.
- Resuspend RNA to a concentration of 2 µg/µL in DEPC-treated H$_2$O.
2.5 Analytical methods

2.5.1 DNA and RNA blots

2.5.1.1 Preparation of radioactive probes (random primer labeling)

The synthesis of the radioactive probe was done with a DNA polymerase (Klenow fragment). The polymerase is primed by random hexanucleotide primers. In the deoxynucleotide triphosphate (dNTP) mix the deoxycytidine triphosphate (dCTP) has the radioactive $^32$P-phosphor isotope incorporated at its $\alpha$-phosphate ($[^{\alpha-32}\text{P}]$-dCTP), which gives a high energy $\beta$-particle emission when the isotope decays (half life 14.3 days).

Procedure:
- Work with high energy radioactive material should only be done by authorized personal, and with proper shielding and protective measures.
- Prepare a DNA template for the radioactive probe.
- Mix in a 1.5 mL microfuge tube 10.5 µL sterile dH$_2$O, and 2 µL template DNA [100-200 ng/µL].
- Denature in boiling water for at least 5 minutes.
- Add 5 µL cold labeling buffer (see Appendix 4), 2.5 µL BSA [4 mg/mL], 1.5 µL dNTP mixture (deoxyadenosine triphosphate, deoxythymidine triphosphate, deoxyguanosine triphosphate [1 mM of each, mixed 1:1:1])
- Add 2.5 µL (25 microcurie (µCi)) $[^{\alpha-32}\text{P}]$-dCTP, and mix with a pipette.
- Add 1 µL DNA polymerase I, (Klenow fragment) [2 units/mL] (NEB).
- Spin briefly and incubate at room temperature for 3 hours.
- Store at -20 °C.

2.5.1.2 Hybridizing sample DNA and RNA with radioactive probe

This hybridization protocol is used for hybridizing radioactive probes made by random primer labeling.

Procedure:
- Put the hybridization buffer at a 65 °C water bath to preheat the buffer.
- Wash blotting membrane with sample DNA in dH$_2$O.
- Put membrane into a hybridization tube. Add 1 mL of hybridization buffer, and incubate at 65 °C on a rotating device for 15 minutes.
- Discard the hybridization buffer. Add 0.1 mL hybridization buffer per square cm of blotting membrane.
- Add random primer labeled probe at a ratio of 1/2000 the amount of hybridization buffer (for example 10 ml hybridization buffer requires 5 µL probe).
- Incubate for about 24 hours at 65°C on a rotating device.
- Dispose of the radioactive hybridization buffer properly.
- Put the wash buffer #1 and #2 at a 65 °C water bath to have hot buffers.
- Wash membrane at 65°C on a rotating device. Twice with wash buffer #1 for 5 minutes, and then seven times for 5 minutes in wash buffer #2. Do a final wash for 20 minutes in wash buffer #2.
- Wrap membrane in plastic wrap, with the side with DNA having a smooth surface. Check radioactivity with a Geiger counter.
- Visualize the result of the hybridization by autoradiography.

2.5.1.3 Autoradiography with radioactive membranes

Autoradiography was done with the Kodak® BioMax® MS equipment and solutions.

- In a dark room, fix the blotting membrane with tape to the autoradiography casing, and insert a x-ray film with intensifying screen. Close the casing.
- Expose film at -80 °C using for a few hours, or overnight, depending on how fresh the radioactive probe is, and how much hybridization occurred.
- Develop the film in a dark room by dipping the film for 1 minute into developer solution, rinse in H₂O, then dip the film in fixer solution for 1 minute.
- Rinse the film in plenty of water, and dry. Take photo with digital camera.

2.5.1.4 Aligning agarose gel photo to autoradiographic films

Procedure:
- Take a photo of the agarose gel with ladder, or rRNA bands, making sure that both top and bottom of gel is included for reference points.
- When continuing the blotting procedure, make sure that the bottom of the blotting membrane is aligned with the bottom of the agarose gel.
- Continue the blotting procedure until a film is developed.
- Mark the point on the film that was the bottom of the blotting membrane, and the point 8.5 cm above that (since an agarose gel is 8.5 cm). This gives top and bottom reference points. Photograph the film.
- On the computer align the bottom point of the gel to the bottom point of the film, and the same for the top point.
2.5.1.5 Slot blot

Procedure:
- Add 3 µL NaOH [5 N] to a 1.5 µL microfuge tube.
- Add 500 ng sample DNA dissolved in H₂O, and if necessary add sterile dH₂O to raise the total volume to 50 µL.
- Denature by incubating at 65 °C for 45 minutes. Cool down to room temperature.
- Add 50 µL saline-sodium citrate buffer (SSC) [20x].
- Setup the dot blot apparatus (PR 600 SlotBlot, Hoefer Scientific Instruments (USA) according to operation instructions.
- Soak blotting membrane for 5 minutes in dH₂O, then for 5 minutes in SSC [10x].
- Load blotting membrane into dot blot apparatus, and pull 200 µL SSC [10x] through sample slot by suctioning with a vacuum aspirator.
- Turn off suction, and add 100 µL SSC [10x] in sample slot. Add denatured DNA sample and mix with a pipette in the slot. Turn on suction and wait until all liquid has been sucked through.
- Turn off suction, and wash slot by sucking through 200 µL SSC [10x].
- Take out the blotting membrane, wrap it into plastic wrap, and crosslink DNA to membrane with CL-1000 Ultraviolet Crosslinker, UVP (USA), set to 1500 energy.
- Hybridize the membrane with a radioactive probe and develop by autoradiography

2.5.1.6 Southern blot

Procedure:
- Isolate genomic DNA
- Digest in a microfuge tube 1.5 µg of genomic DNA in 20 µL with 30 units enzyme for 4 hours.
- Ethanol precipitate the digested DNA.
- Resuspend DNA in 20 µL, including DNA gel loading buffer.
- Run reaction on an agarose gel.
- Check gel under UV-light. Take a photo with a digital camera.
- Transfer DNA to a nylon membrane according to protocol.
- Put membrane into SSC [2x]. Check gel under UV-light for complete transfer.
- Wrap membrane into plastic wrap, and crosslink DNA to membrane with CL-1000 Ultraviolet Crosslinker, UVP (USA), set to 1500 energy.
- Hybridize the membrane with a radioactive probe and develop by autoradiography
2.5.1.7 Northern blot

Procedure:
- All equipment and solutions used have to be RNase-free.
- Sterilize an electrophoresis chamber with tray and comb with 3 % H₂O₂ overnight.
- Dissolve 0.78 g agarose in 37 mL DEPC-treated water (1.3 % agarose gel). Cool down in water bath at 65 °C.
- Add 12 mL MOPS buffer [5x], and 11 mL formaldehyde [37 %].
- Mix gently by swirling and pour into a gel tray. Put in comb.
- Prepare the samples by mixing for each sample in a microfuge tube:
  - 2.5 µL DEPC-treated water
  - 2.0 µL MOPS buffer [5x]
  - 3.5 µL formaldehyde
  - 3.5 µL ethidium bromide [100 µg/mL]
  - 10.0 µL formamide
  - 2.0 µL RNA sample [2 µg/µL]
- Incubate samples at 65 °C for 15 minutes.
- Put the gel into the electrophoresis chamber and add running buffer to cover gel (315 mL DEPC H₂O, 90 ml MOPS [5x], and 45 mL formaldehyde).
- Prerun for 5 minutes at 60 mA.
- Cool down samples, spin in centrifuge at max speed for 1 minute, and add 2 µL RNA gel loading buffer.
- Mix with a pipette and add 20 µL sample per well.
- Run the samples at 60 mA for 10 minutes in reverse direction, then in normal direction until the bromphenol blue band is at the bottom of the gel.
- Photograph the gel with a digital camera.
- Wash briefly in DEPC-treated water and transfer RNA to a nylon membrane following the protocol.
- Transfer for 5.5 hours. Put membrane into SSC [2x]. Check gel under UV-light for complete transfer.
- Wrap membrane into plastic wrap, and crosslink DNA to membrane with CL-1000 Ultraviolet Crosslinker, UVP (USA), set to 1500 energy.
- Hybridize the membrane with a radioactive probe and develop by autoradiography.

2.5.2 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

For the mass spectrometry experiment a Criterion XT 4-12 % Bis Tris Precast gel (BioRad) catalog #345-0123 was used according to supplier’s instructions.

The Mini-Protean 3 (BioRad) gel casting system was used for making the 12 % polyacrylamide gels. For separating gel: 2.85 mL dH₂O, 3.3 mL solution A, 2.06 mL solution B, 32.5 µL Ammonium persulfate [10 %]. For the stacking gel: 3.61 mL dH₂O, 800 µL solution A, 1.5 mL solution B’, 60 µL Ammonium persulfate [10 %]). For larger gels a prefabricated gel was used (BioRad).
 Procedure:
- Grow *Chlamydomonas* with air bubbling to a high density.
- Centrifuge 40 mL of cell culture at 6,000 g for 5 minutes.
- Resuspend the pellet in a minimal amount of dH2O.
- Add 3X SDS-PAGE sample buffer to a final concentration of 1X.
- Store sample at -20 °C, or use immediately.
- Run sample on the gel, 80 volts for 20 minutes, then 120 volts until desired. For sharper bands, run at lower voltage.
- Stain sample with gel staining solution for 15 minutes with shaking.
- Destain with destaining solution overnight with shaking.
- Store gel in dH2O.

2.5.3 Mass spectrometry

In-gel digest procedure (from Anders Moen, UiO):
- Run a SDS-PAGE gel with the samples.
- Cut out the relevant band, and cut it to smaller pieces, in a sterile hood.
- Transfer the pieces to 1.5 mL microfuge tubes.
- Add 500 μL isopropanol and 500 μL dH2O to each tube.
- Incubate the gel pieces in isopropanol and dH2O for 30 min at 55 °C.
- Remove supernatant, and repeat washing with isopropanol/water until gel pieces are blank. Remove solution.
- Add 100 μL 100 % isopropanol to each tube and incubate at room temperature for 15 minutes until the gel pieces become white. Remove the solution.
- Add 100 μL DTT solution to all tubes and incubate for 1 hour at 56 °C.
- Remove solution and add 100 μL iodacetamide solution and incubate in dark for 1 hour.
- Remove solution and wash three times with 100 μL acetonitrile.
- Add 30 μL 16 ng/μL trypsin solution, or enough for covering the gel pieces.
- Incubate the gel pieces with trypsin for 30 minutes, until the gel pieces are blank again, on ice.
- Remove trypsin solution.
- Add 100 μL 50 mM ammonium bicarbonate, or enough to cover the gel pieces.
- Incubate overnight at 37 °C
- Add 30 μL 5% formic acid, and 30 μL 100% acetonitrile to each tube.
- Incubate at 37 °C for 20 minutes.
- Transfer supernatant (containing peptides) to a new microfuge tube.
- Extract peptides from gel pieces one more times with 30 μL 5% formic acid, and 30 μL 100% acetonitrile.
- Extract remaining gel pieces with 60 μL acetonitrile, by incubating at 37 °C for 20 minutes.
- Transfer solution to the new microfuge tube.
- Dry the solution in a speedvac.
Nanoflow On-line Liquid Chromatographic MS Analysis of Proteolytic Peptides
(from Anders Moen, UiO):

Reverse phase (C18) nano online liquid chromatographic MS/MS analyses of proteolytic peptides were performed using a HPLC system consisting of two Agilent 1200 HPLC binary pumps (nano and capillary) with corresponding autosampler, column heater and integrated switching valve. This LC system was coupled via a nanoelectrospray ion source to a LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). For the analyses, 1 μl of peptide solution was injected onto the 5 x 0.3-mm extraction column filled with Zorbax 300 SB-C18 of 5 μm particle size (Agilent). Samples were washed with a mobile phase of 97% formic acid/3% acetonitrile. The flow rate was 10 μl/min provided by the capillary pump. After 7 min, the switching valve of the integrated switching valve was activated, and the peptides were eluted in the back-flush mode from the extraction column onto the 150 × 0.075-mm C18, 3 μm resin, column (GlycproSIL C18–80Å, Glycpromass, Stove, Germany). The mobile phase consisted of acetonitrile and MS grade water, both containing 0.1% formic acid. Chromatographic separation was achieved using a binary gradient from 10 to 55% of acetonitrile in 60 or 210 min. The flow rate was 0.2 μl min\(^{-1}\) provided by the nanoflow pump.

Mass spectra were acquired in the positive ion mode applying a data-dependent automatic switch between survey scan and tandem mass spectra (MS/MS) acquisition. Peptide samples were analyzed with a collision induced dissociation (CID) fragmentation method, acquiring one Orbitrap survey scan in the mass range of \(m/z\) 200–2000 followed by MS/MS of the six most intense ions in the Orbitrap. The target value in the LTQ-Orbitrap was 1,000,000 for survey scan at a resolution of 30,000 at \(m/z\) 400 using lock masses for recalibration to improve the mass accuracy of precursor ions. Ion selection threshold was 500 counts. Selected sequenced ions were dynamically excluded for 180 s.

Data Analysis (from Anders Moen, UiO):

Mass spectrometric data were analyzed with the in-house maintained Ectoin and Uniprot protein sequence database using SEQUEST™. The mass tolerances of a fragment ion and a parent ion were set to 0.5 Da and 10 ppm, respectively. Methionine oxidation and cysteine carbamidomethylation were selected as a variable or fixed modification. A false discovery rate of 0.01 was required for proteins and peptides with a minimum length of 6 amino acids.

2.5.4 High pressure liquid chromatography (HPLC)

HPLC was performed in the lab of Dr. Galinski (Germany), with an apparatus set up for the detection of ectoine. A lyophilized sample of the ectA transformant was sent for analysis.
2.5.5 Salt tolerance experiments

Solutions of high salt (HS) media with 1.5 % agar were made with the following NaCl concentrations (w/w): 0.9, 1.0, 1.1, 1.2, 1.4, and 1.6. Solutions were autoclaved and poured to sterile plates. When solid, plates were plated with 100 μL of *Chlamydomonas* cell culture at a density of ~50 million cells/mL (cells were centrifuged and resuspended to get to the required density). After plating, plates were exposed to light for about 20 days before documenting the results.
3.0 Results

3.1 Making the *ectA* transformation vector construct

3.1.1 *ectA* transformation vector overview

A plasmid vector capable of stably integrating a functional *ectA* transgene was constructed by using the backbone of a *Chlamydomonas* chloroplast transformation vector previously created by Blowers *et al.* (1993) (plasmid pCrc_32). The vector is capable of integrating transgenes in a site specific manner, and is using essentially the same construct design as Blowers *et al.* (1989) (figure 1.4) used; the 5.3 kb chloroplast BamHI-EcoRI fragment provides homologous flanking sequences, into which the transgene is inserted. The *ectA* coding sequence was provided on a plasmid containing the *Pseudomonas stutzeri ectABCD* operon (p_ect_operon_stutzeri), provided by Dr. Galinski (Seip *et al.*, 2011).

The vector created has the *ectA* coding sequence flanked by the 5' and 3' regions of the *Chlamydomonas* chloroplast genes *rbcL* (rubisco large subunit) and *psaB* (D1 protein of photosystem I), respectively, providing necessary elements for transcription and translation (figure 3.1). This chimeric gene is inserted about midway into the ~5.3 kb BamHI-EcoRI *Chlamydomonas* wild type fragment, splitting the ~5.3 kb fragment into a ~2.3 kb, and a ~3.0 kb fragment. The ~3.0 kb fragment contains the full length *atpB* gene, allowing selection of positive transformants by restored photosynthesis function in *atpB* deletion mutants. In the pCrc_32 vector backbone the BamHI-EcoRI fragment is cloned into BamHI/EcoRI sites in a pUC8 vector, making the total size of the transformation vector ~9.2 kb.

![Figure 3.1 Overview of the transformation vector for targeted insertion of *ectA* into the *Chlamydomonas* chloroplast genome](image_url) 

*Figure 3.1 Overview of the transformation vector for targeted insertion of *ectA* into the *Chlamydomonas* chloroplast genome.* The total size of the vector is a few base pairs more than the sum of the sizes indicated, due to some short sequences from cloning steps.
3.1.2 Subcloning of *ectA*

Subcloning of *ectA* was essentially done in three steps (figure 3.2):

First, a PCR was done on p_ect_operon_stutzeri. The primers used (pcr5’, and pcr3’) amplified a product containing the *ectA* coding sequence, with primer-designed flanking restriction enzyme sites: NdeI in the 5’ end, and NheI plus SpeI in the 3’ end. (The reason for putting two restriction sites in the 3’ end of the PCR product is that the sites would be used for cloning into Xbal sites in later steps (they produce compatible ends with Xbal), however; Xbal itself could not be used since an XbaI site is present in the coding sequence of *ectA.*) The resulting 631 bp amplification product was purified from an agarose gel, and digested with NdeI and SpeI.

Second, the PCR fragment was cloned into NdeI and XbaI sites of plasmid SK+_157_NdeI to create plasmid SK+_ectA. This step fused *ectA* to the 5’ region of *rbcL*. The plasmid was transformed into competent *E. coli* TB1 cells. Positive transformants were selected for maxi preparation.

Third, SK+_ectA was partially digested with NheI, because of an additional NheI site present in the SK+_157_NdeI backbone sequence. The resulting ~3800 bp fragment was purified from a gel, and subsequently fully digested with XhoI. This yielded a fragment of 772 bp which was subsequently cloned into XhoI and XbaI sites of plasmid pCrc32 (which contains the *psaB* 3’ region, BamHI-EcoRI fragment, and pUC8 sequence), to create plasmid pCrc32_ectA. This was transformed into competent *E. coli* TB1 cells, and positive transformants were selected for maxi preparation.

Sequencing of the pCrc_32_ectA plasmid was done covering the *ectA* sequence and parts of the flanking 5’- and 3’ regions. The results indicated that the cloning process had proceeded as intended, and without any mutations.
Figure 3.2 Subcloning of ectA. The ectA fragment (green) was cloned into SK+157_Nde (which contains the \textit{rbcL} 5’ region, yellow). The 772 bp fragment (containing the \textit{rbcL} 5’ region + ectA) was then cloned into pCrc_32 (which contains the \textit{psaB} 3’ region (blue), BamHI-EcoRI fragment (orange), and pUC8 sequence (grey)).

3.2 Transformation and selection of \textit{ectA} transformants

3.2.1 Microprojectile bombardment and phototrophic selection

The \textit{Chlamydomonas} atpB deletion mutant was bombarded with pCrc_32_ectA plasmids coated onto gold microprojectiles. After bombardment, the cells were transferred to high salt (HS) plates for phototrophic selection. Individual colonies of \textit{Chlamydomonas} were visible after 2-3 weeks.
3.2.2 DNA slot blot

Colonies were picked from selection plates, grown in liquid HS medium, and total DNA was isolated. The DNA was used for slot blot analysis, testing for the presence of the *ectA* sequence, by a radioactively labeled probe prepared from the complete *ectA* coding sequence (figure 3.3). 500 ng of DNA from each culture was loaded into its respective slot. Slot blotting done with DNA from eleven transformants revealed a strong signal for two of the DNAs. This showed that *ectA*, or large parts of the *ectA* sequence, has integrated into the genome of two out of eleven analyzed DNAs. Transformant #1 showed the strongest signal, and consequently this culture was selected for further analysis.

3.3 Analysis of transformant

3.3.1 Southern blot

Using the DNA isolated from transformant #1 a DNA gel blot (Southern blot) was performed, in order to determine how many chloroplast chromosome copies had been transformed (homoplasmicity), and if the integration had occurred as expected.

DNA from the *ectA* transformant (#1), transformation vector (V), and the *atpB* deletion mutant (del), were digested with BamHI and EcoRI, and loaded onto an agarose gel (figure 3.5 (A)). Depending on the state of a chloroplast chromosome it was expected to produce a specific size of the BamHI-EcoRI fragment, indicating if the chromosome was wild type, transformed, or had a deletion (figure 3.4). A radioactively labeled probe was made from a part of the 5.3 kb BamHI-EcoRI fragment: a ~741 bp SnaBI-EcoRI fragment (indicated in grey in figure 3.4), and was expected to bind to all three versions of the chromosomes.

After blotting the DNA onto a blotting membrane and hybridizing to the labeled probe, the hybridization signal could be visualized with autoradiography (figure 3.5 (B)). The autoradiography clearly shows that transformant #1 contains chromosomes with the expected size for *ectA* transformants (4.0 kb). Compared to the signal for chromosomes with wild type sequence (5.3 kb) it appears that the ratio of transformed:wild type chromosomes are ~1:1. Presumably, during the
transformation procedure, a certain number of chloroplast chromosomes integrate the complete \textit{atpB} gene without incorporating \textit{ectA}. There are also some faint bands in the transformant #1 lane, which are probably a result the probe binding to similar sequences found elsewhere in the genomic DNA. Unexpectedly however; no strong signal was detected from the deletion mutant. The weak signal might have been due a difference in sample loading on the agarose gel. A strong signal became visible only after exposing an autoradiography film for 3.5 times longer than the first exposure (2 vs. 7 days). The signal did however appear around the expected size (2.9 kb), and this signal was not visible in transformant #1, even after 7 days exposure (data not shown). This indicates that there are no deletion type chromosomes in the transformant chloroplast.

Figure 3.4 DNA cut with BamHI-EcoRI are expected to produce different sizes depending on its transformation state. The expected sizes are indicated with the underscored number above each DNA type. The grey area on the right side of each DNA type indicates the ~741 SnaBI-EcoRI fragment that was used as a radioactively labeled probe. The transformant DNA produces a 4.0 kb fragment because of a EcoRI site in the \textit{ectA} sequence (indicated).

Figure 3.5 (A) Agarose gel of BamHI-EcoRI digested DNA, and (B) Southern blot of BamHI-EcoRI digested DNA hybridized to a probe prepared from a ~741 bp SnaBI-EcoRI fragment. In (B) the “del” sample had to be exposed 3.5 times longer than “#1” and “V”, in order to get a strong signal. The longer exposure did not reveal any extra bands on #1 or “V”. Of the “#1”, and “del” sample 1.5 µg was loaded, of the “V” sample 1 ng was loaded. L = ladder, #1 = transformant #1 DNA, V = transformation vector DNA, del = \textit{atpB} deletion mutant DNA.
Because there was an EcoRI site within the *ectA* sequence, an additional Southern blot had to be done in order to investigate if the full length chimeric *ectA* with 5’ and 3’ regions had inserted. This was done by digesting transformant #1 DNA, and the transformation vector with KpnI and HindIII. The expected size of the relevant fragments were ~2.8 for the wild type chromosome and ~4.2 for the transformed chromosome (figure 3.6). A radioactively labeled probe was prepared from a portion of the *atpB* gene that was amplified by PCR (grey area in figure 3.7). The DNA was run on a gel, transferred to a blotting membrane, hybridized with the *atpB* probe, and the result was visualized by autoradiography (figure 3.8). The result shows that there are chromosomes in the transformant of the correct size for the *ectA* with 5’ and 3’ regions, by comparing to the positive vector control or the DNA ladder. This Southern blot also indicates that the ratio of transformed:wild type chromosomes is ~1:1

![Figure 3.6 DNA cut with KpnI-HindIII produce different sizes in wild type and transformant DNA.](image)

**Figure 3.6 DNA cut with KpnI-HindIII produce different sizes in wild type and transformant DNA.** The expected sizes are indicated with the underscored number above each DNA type. Note that the chimeric *ectA* is cloned into the rightmost KpnI site, destroying it in the process. The grey area indicates the *atpB* fragment that was used as a radioactively labeled probe.

![Figure 3.7 Southern blot of KpnI-HindIII digested DNA, hybridized to an atpB probe.](image)

**Figure 3.7 Southern blot of KpnI-HindIII digested DNA, hybridized to an atpB probe.** L = ladder, #1 = transformant #1 DNA, V = transformation vector DNA
3.3.2 Northern blot

After confirming that the ectA construct with 3’- and 5’-regions had inserted properly, RNA gel blots (northern blot) were done in order to evaluate the extent of ectA transcripts. Previous reports on chimeric transgenes with the rbcL 5’ region have shown that the transcripts are sensitive to light degradation (Salvador et al., 1993). Thus, total RNA was isolated from transformant #1 grown at both light and dark conditions. The samples were isolated from cells in the exponential phase grown on a 12 hour/12 hour light/dark cycle. One sample was isolated in the dark, after a period of 11 hours of darkness; the other sample was isolated after a period of 2.5 hours of light. Control samples were also isolated from a Chlamydomonas culture transformed with a different, unrelated gene, and were also isolated using light/dark conditions. ~2.5 µg of total RNA from each of the samples were run on an agarose gel (figure 3.8 (A)).

The gel was then blotted to a membrane which was hybridized with a radioactively labeled probe prepared from the ectA coding region (figure 3.8 (B)). The result showed that only RNA from transformant #1 isolated in the dark showed a strong hybridization signal after ~1 day exposure. This indicates that the ectA is transcribed from its rbcL promoter, but accumulates to a lower level compared to the light exposed sample. Previous reports attribute this phenomenon to an increased rate of transcript degradation in light rather than a decrease of transcription (Salvador et al., 1993).

In order to evaluate the relative abundance of the ectA transcript, the blotting membrane was hybridized with an additional probe prepared from a PCR amplified ~1000 bp fragment covering the atpB gene (figure 3.9). The atpB gene is a highly transcribed endogenous “household” gene and can serve as comparison for the relative levels of transcript. The result of the autoradiography shows that the level of atpB transcript is approximately equal in the transformant and the wild type (except for sample “NC_L”, due to less total RNA being loaded), making it usable as a comparison-transcript. From the result it is clear that ectA transcripts accumulate to a much lower level than atpB. However, since the radioactively labeled probes differ in size by a factor of about 1.67 (1000 bp/600 bp), the intensity of the ectA have to be multiplied by this factor for a direct comparison. Still, the difference is quite large.
Due to the short exposure time (1 day) of the initial northern blot experiment (figure 3.8), no ectA transcript could be detected in the light-condition isolated sample (“#1L”). A new northern blot experiment was therefore performed, using the same samples and ectA probe, but with 5 days of exposure time (figure 3.10). The experiment revealed a strong signal also for the “#1L” sample, showing that ectA transcripts accumulate also in the light, but visibly less than in the dark, even with the agarose gel (figure 3.10 (A)) showing that “#1L” had more RNA loaded. Furthermore, it can be seen that degradation-product signals are stronger in the “#1L” sample (the smear below the main band), confirming that degradation is intensified during light conditions.

Figure 3.8 (A) Agarose gel of total RNA from ectA transformant and control samples, in light and dark conditions, and (B) the same gel northern blotted and hybridized to an ectA probe for ~1 day. The strong bands in (A) are ribosomal RNA from the different organelles. “#1L,” and “NC_L” has visibly less loaded than the other samples, due to a loading problem. #1D=ectA transformant RNA isolated in the dark, NC_D= negative control RNA isolated in the dark, NC_L=negative control RNA isolated in the light, #1L= ectA transformant RNA isolated in light.

Figure 3.9 Northern blot, hybridized to ectA and atpB probe for ~1 day. By comparing the ectA signal with the atpB signal it is clear that ectA transcripts accumulate much less than atpB transcripts. See legend to figure 3.8 for details.
3.3.3 SDS-PAGE

A SDS-PAGE experiment was done to detect the EctA protein. Considering that transcript accumulation of ectA was at a low level the experiment was not expected to reveal readily identifiable EctA band. Nevertheless, protein samples were prepared (as described in Materials and Methods) from exponentially growing algae that had been growing for 11 hours in the dark, or 6 hours in the light. Control samples were prepared from wild type algae under the same conditions. The samples were run on a 12 % polyacrylamide gel (figure 3.11). EctA has a theoretical mass of 21.5 kDa. A band around that size was more prominent in the transformant-dark than in the control-dark sample, however loading inequalities could be the cause. Moreover, the same band is also prominent in the control-light sample, indicating that this is a normal band in wild type algae, at least in light. It was therefore no strong indication of EctA accumulation by this experiment.

Figure 3.11 12 % SDS-PAGE Mini-Protean 3 (BioRad) cast gel loaded with protein samples from transformant and wild type algae, grown at 11 hours in the dark, or 6 hours in the light. The figure shows only the relevant part of the gel. L=protein ladder, #1D=ectA transformant protein isolated in the dark, WT_D= wild type alga protein isolated in the dark, WT_L= wild type alga protein isolated in the light, #1_L= ectA transformant protein isolated in light.
3.3.4 Mass spectrometry

In an effort to raise the detection limits of EctA, a mass spectrometry (MS) experiment was performed. The two protein samples described (chapter 3.3.3) were maximally loaded on a precast SDS-PAGE gel, and after electrophoresis, two pieces of the gel ranging from ~23 kilo Dalton (kDa) to ~18 kDa were cut out, treated with the proteolytic enzyme trypsin, and otherwise prepared for mass spectrometry analysis as described in Materials and Methods (figure 3.12).

The mass spectrometry was carried out as described in Materials and Methods. The data generated was analyzed with the Uniprot (http://www.uniprot.org/) protein sequence database using SEQUEST™, and indicated that the samples contained a large number of wild type *Chlamydomonas* proteins of the expected size (Appendix 2). However, no indication of EctA was shown. A specific search using SEQUEST™ with only EctA in the SEQUEST™ database did not produce any matching masses either.

![Figure 3.12 Criterion XT precast 4-12 % Bis Tris SDS-PAGE gel with protein samples from transformant isolated after 11 hours in the dark (#1D), and after 6 hours in the light (#1L). The white squares indicate the areas that were cut out of the gel for analysis by mass spectrometry. The cuts were not perfectly identical due to human error.](image)

3.3.5 High pressure liquid chromatography (HPLC)

A lyophilized sample of the *ectA* transformant was sent to Dr. Galinski’s laboratory for evaluation of ectoine accumulation in the algae by HPLC. They reported that no ectoine could be detected in the sample by the methods employed.
3.3.6 Salt tolerance experiments

An experiment was carried out where wild type algae, and the *ectA* transformed algae were subjected to phototrophic growth on high salt (HS) plates with varying NaCl concentrations, ranging from 0 – 1.8 % NaCl (w/w). It was found that the *ectA* transformants could not support growth on plates with more than ~1.1 % NaCl content, whereas the wild type algae could not support growth on plates with more than ~1.2 % NaCl content (figure 3.13), indicating no increased salt tolerance of the *ectA* transformant.

Figure 3.13. *ectA* transformant (#1) and wild type (WT) algae grown phototrophically on HS plates with varying NaCl concentration (shown as % NaCl w/w). Cells were grown ~20 days prior to phototography.

3.4 Making a codon optimized *ectA* transformation vector

3.4.1 Codon optimization of *ectA*

The expression of foreign genes in the *Chlamydomonas* chloroplast is greatly reduced when using a transgene that has a radically different codon usage than the *Chlamydomonas* chloroplast (Potvin and Zhang, 2010). Analysis of codon usage in the *ectA* gene of *Pseudomonas stutzeri* versus the *Chlamydomonas* chloroplast usage was done with the only “Graphical codon usage analyzer” web-tool (http://gcua.schoedl.de/index.html) (Fuhrmann et al., 2004). The results showed that the codon usage difference was severe (Appendix 1).

To make an *ectA* gene with a codon bias similar to the *Chlamydomonas* chloroplast a codon optimization procedure was carried out as described in Materials and Methods. The result of the optimization process yielded a sequence named *OectA* (figure 3.14).
<table>
<thead>
<tr>
<th>Met</th>
<th>Pro</th>
<th>Thr</th>
<th>Leu</th>
<th>Lys</th>
<th>Arg</th>
<th>Asn</th>
<th>Ser</th>
<th>Ile</th>
<th>Asn</th>
<th>Asn</th>
<th>Pro</th>
<th>Lys</th>
<th>Gly</th>
<th>Ile</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ATG</td>
<td>CCT</td>
<td>TCA</td>
<td>TTA</td>
<td>AAA</td>
<td>CAT</td>
<td>TCA</td>
<td>TTT</td>
<td>AAC</td>
<td>AAC</td>
<td>CCA</td>
<td>AAA</td>
<td>GGT</td>
<td>ATT</td>
</tr>
<tr>
<td>1</td>
<td>ATG</td>
<td>CCT</td>
<td>ACC</td>
<td>CTA</td>
<td>AAA</td>
<td>AGG</td>
<td>TCA</td>
<td>ATC</td>
<td>AAC</td>
<td>ACC</td>
<td>CCC</td>
<td>AAA</td>
<td>GGC</td>
<td>ATT</td>
</tr>
<tr>
<td></td>
<td>Val</td>
<td>Leu</td>
<td>Ser</td>
<td>Phe</td>
<td>Pro</td>
<td>Thr</td>
<td>Val</td>
<td>Met</td>
<td>Leu</td>
<td>Arg</td>
<td>Arg</td>
<td>Pro</td>
<td>Thr</td>
<td>Asp</td>
</tr>
<tr>
<td>46</td>
<td>GGT</td>
<td>GTC</td>
<td>AGT</td>
<td>TAA</td>
<td>CAT</td>
<td>CCA</td>
<td>AGA</td>
<td>GTA</td>
<td>TTA</td>
<td>GTG</td>
<td>CTT</td>
<td>GTA</td>
<td>GAT</td>
<td>CTT</td>
</tr>
<tr>
<td>46</td>
<td>GGT</td>
<td>TGG</td>
<td>AGT</td>
<td>TCC</td>
<td>ACC</td>
<td>GTA</td>
<td>ATG</td>
<td>TCT</td>
<td>CGT</td>
<td>CTT</td>
<td>CCA</td>
<td>ACC</td>
<td>ACC</td>
<td>GGC</td>
</tr>
<tr>
<td></td>
<td>Asp</td>
<td>Gly</td>
<td>Tyr</td>
<td>Asn</td>
<td>Ser</td>
<td>Val</td>
<td>Tyr</td>
<td>Cys</td>
<td>Asn</td>
<td>Leu</td>
<td>Leu</td>
<td>Gin</td>
<td>Cys</td>
<td>Ser</td>
</tr>
<tr>
<td>136</td>
<td>GTC</td>
<td>GAA</td>
<td>ACA</td>
<td>GCC</td>
<td>ATC</td>
<td>CAA</td>
<td>GCT</td>
<td>GCC</td>
<td>GCA</td>
<td>AAC</td>
<td>CAA</td>
<td>GCT</td>
<td>AAA</td>
<td>AAT</td>
</tr>
<tr>
<td>136</td>
<td>GAC</td>
<td>GGT</td>
<td>TAC</td>
<td>ACA</td>
<td>CAC</td>
<td>ACG</td>
<td>CTG</td>
<td>TTC</td>
<td>GCC</td>
<td>GGC</td>
<td>CCG</td>
<td>CCG</td>
<td>TTC</td>
<td>GTC</td>
</tr>
<tr>
<td></td>
<td>Ala</td>
<td>Asp</td>
<td>Thr</td>
<td>Ala</td>
<td>Ile</td>
<td>Ala</td>
<td>Ala</td>
<td>Glu</td>
<td>Ala</td>
<td>Asn</td>
<td>Ala</td>
<td>Glu</td>
<td>Gly</td>
<td>Leu</td>
</tr>
<tr>
<td>226</td>
<td>GCT</td>
<td>GCT</td>
<td>TGG</td>
<td>AGT</td>
<td>TCA</td>
<td>CAT</td>
<td>TAA</td>
<td>GTA</td>
<td>CCA</td>
<td>AAG</td>
<td>TCA</td>
<td>GGC</td>
<td>GCA</td>
<td>GCA</td>
</tr>
<tr>
<td>226</td>
<td>GTC</td>
<td>GTC</td>
<td>TGG</td>
<td>CAG</td>
<td>GTC</td>
<td>TAC</td>
<td>CCA</td>
<td>GCC</td>
<td>CCG</td>
<td>CCG</td>
<td>TCG</td>
<td>CCG</td>
<td>GAC</td>
<td>ACG</td>
</tr>
<tr>
<td></td>
<td>Phe</td>
<td>Val</td>
<td>Trp</td>
<td>Gln</td>
<td>Val</td>
<td>Ala</td>
<td>Val</td>
<td>Asp</td>
<td>Ser</td>
<td>Ser</td>
<td>Met</td>
<td>Arg</td>
<td>Gly</td>
<td>Gln</td>
</tr>
<tr>
<td>316</td>
<td>CTT</td>
<td>TCA</td>
<td>AGA</td>
<td>TCA</td>
<td>GCT</td>
<td>GCT</td>
<td>AGT</td>
<td>TCA</td>
<td>TTA</td>
<td>CAA</td>
<td>AGA</td>
<td>TCA</td>
<td>TTA</td>
<td>CAA</td>
</tr>
<tr>
<td>316</td>
<td>CAG</td>
<td>CGT</td>
<td>ATG</td>
<td>TTA</td>
<td>GTA</td>
<td>GTA</td>
<td>GCT</td>
<td>ACA</td>
<td>ATT</td>
<td>ACA</td>
<td>GGT</td>
<td>CTA</td>
<td>GCT</td>
<td>CCA</td>
</tr>
<tr>
<td></td>
<td>Leu</td>
<td>Ala</td>
<td>Leu</td>
<td>Arg</td>
<td>Met</td>
<td>Leu</td>
<td>Leu</td>
<td>Ala</td>
<td>Leu</td>
<td>Thr</td>
<td>Ala</td>
<td>Arg</td>
<td>Val</td>
<td>Ala</td>
</tr>
<tr>
<td>406</td>
<td>GTC</td>
<td>TCA</td>
<td>CAG</td>
<td>CGC</td>
<td>ATG</td>
<td>TTA</td>
<td>TCA</td>
<td>AAG</td>
<td>CCT</td>
<td>TCT</td>
<td>GAC</td>
<td>CGG</td>
<td>TTA</td>
<td>CAT</td>
</tr>
<tr>
<td>406</td>
<td>GGG</td>
<td>GCG</td>
<td>TCA</td>
<td>CAG</td>
<td>GCG</td>
<td>CTG</td>
<td>TCC</td>
<td>AAG</td>
<td>GGC</td>
<td>TTC</td>
<td>GAC</td>
<td>CGC</td>
<td>CTC</td>
<td>GAT</td>
</tr>
<tr>
<td></td>
<td>Ala</td>
<td>Asn</td>
<td>Cys</td>
<td>Thr</td>
<td>Thr</td>
<td>Thr</td>
<td>Thr</td>
<td>Leu</td>
<td>Phe</td>
<td>Ala</td>
<td>Arg</td>
<td>Asp</td>
<td>Thr</td>
<td>His</td>
</tr>
<tr>
<td>496</td>
<td>GCT</td>
<td>GCT</td>
<td>TGG</td>
<td>ACC</td>
<td>ACA</td>
<td>CAT</td>
<td>TTA</td>
<td>CAA</td>
<td>CAT</td>
<td>TTA</td>
<td>CAA</td>
<td>GAT</td>
<td>GAA</td>
<td>TTA</td>
</tr>
<tr>
<td>496</td>
<td>GGC</td>
<td>GTC</td>
<td>TCA</td>
<td>CAG</td>
<td>GAG</td>
<td>GAG</td>
<td>GTC</td>
<td>TAC</td>
<td>GCC</td>
<td>GGC</td>
<td>CCG</td>
<td>GCC</td>
<td>GGC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Thr</td>
<td>Val</td>
<td>Ser</td>
<td>Leu</td>
<td>Gly</td>
<td>Glu</td>
<td>Leu</td>
<td>Lys</td>
<td>Glu</td>
<td>His</td>
<td>Ala</td>
<td>*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>541</td>
<td>AGT</td>
<td>GTC</td>
<td>TCA</td>
<td>TTA</td>
<td>GAA</td>
<td>GAA</td>
<td>CAA</td>
<td>TTA</td>
<td>AAA</td>
<td>AAA</td>
<td>CAT</td>
<td>CCA</td>
<td>TCA</td>
<td>TGA</td>
</tr>
</tbody>
</table>

**Figure 3.14 Codon-optimized ectA sequence (Oecta).** The *Oecta* nucleotide sequence is shown on top of the original *ectA* sequence. Optimized codons in the *Oecta* sequence are highlighted in black. Amino acid abbreviations are shown above the codons.
3.4.2 Subcloning of \textit{OectA}

The \textit{OectA} sequence was synthesized by Life Technologies™, GeneArt®. The sequence was cloned into the \textit{Chlamydomonas} transformation vector creating essentially the same construct as for \textit{ectA} (figure 3.1), with only the \textit{ectA} coding sequence changed to that of the \textit{OectA}. Thus the subcloning procedure was similar to that of \textit{ectA} with some modifications:

Because this coding sequence of \textit{ectA} did not contain an XbaI site (as was the case for \textit{ectA}), an XbaI site was designed flanking the 3’ end of the \textit{OectA} sequence. However, the XbaI site was overlapping with both upstream and downstream GATC sequences, in which the adenine in most \textit{E. coli} strains are methylated, rendering the XbaI enzyme inactive. Consequently \textit{E. coli dam-}/
\textit{dcm-} cells (NEB) had to be used in all but the last subcloning step of \textit{OectA}, since this strain does not methylate the GATC sequence.

Subcloning of \textit{OectA} was done essentially in three steps (figure 3.15):

First, the synthesized plasmid, geneart\textunderscore\textit{OectA} was transformed into competent \textit{E. coli dam-}/
\textit{dcm-} cells, and a maxi preparation was performed. The plasmid was then digested with NdeI and XbaI, and the resulting 584 bp fragment was isolated from a gel.

Second, the 584 bp fragment was cloned into NdeI and XbaI sites of plasmid SK+\textunderscore\textit{157\textunderscoreNdeI}, to create plasmid SK+\textunderscore\textit{OectA}. This was transformed into competent \textit{E. coli dam-}/
\textit{dcm-} cells, and positive transformants were selected for maxi preparation.

Third, SK+\textunderscore\textit{OectA} was digested with XhoI and XbaI and the released 746 bp fragment was cloned into XhoI and XbaI sites of plasmid pCrc32 to create plasmid pCrc32\textunderscore\textit{OectA}. This was transformed into competent \textit{E. coli TB1} cells, and positive transformants were selected for maxi preparation.

Sequencing of the pCrc32\textunderscore\textit{OectA} plasmid was done covering the \textit{OectA} sequence and parts of the flanking 5’- and 3’ regions. The results indicated that the cloning process had proceeded as intended. However, sequencing revealed that a thymidine nucleotide had been deleted 36 nucleotides upstream of the start codon of \textit{OectA}, thus being a part of the 5’ UTR. The deletion was however suspected to be of minor importance as it was not a part of the ribosome binding site.
3.5 Tranformation and selection of OectA transformants

3.5.1 Microprojectile bombardment and phototrophic selection

The Chlamydomonas atpB deletion mutant was bombarded with pCrc_32_OectA plasmid coated onto gold microprojectiles. After bombardment, the cells were transferred to high salt (HS) plates for phototrophic selection. Unexpectedly, only two colonies appeared after a few weeks. The bombardment procedure was repeated, two additional times, bombarding in total ~12 plates. This did not yield any additional phototrophic colonies. One plate was also bombarded
with another transformation vector also containing the full length \( atpB \) gene and homologous sequences, but puzzlingly this plate did neither yield any colonies. As a control for the transformation procedure an aliquot of the \( OecA \) transformation vector was given to a lab in Spain, and they performed a microprojectile bombardment. They were able to get two colonies after this procedure, and the colonies were sent back to us for analysis.

3.5.2 DNA slot blot

Cells from the four colonies were grown in liquid culture, and genomic DNA was isolated from them. A DNA slot blot was performed, testing for the presence of the \( OecA \) sequence, by a radioactively labeled probe prepared from the complete \( OecA \) coding sequence (figure 3.16). A positive control sample of the transformation vector was also blotted. The result of the experiment showed that none of the four transformants contained the \( OecA \) sequence. Unfortunately, no transformants were obtained.

![Figure 3.16 Transformant DNA slot blotted to a radioactively labeled \( OecA \) probe. #1 is the positive control sample. #2, #3 are samples from Spain, #4 and #5 are locally isolated samples.](image)
4.0 Discussion

The *ectA* gene was stably transformed into the *Chlamydomonas* chloroplast in order to investigate the possibility of engineered expression of ectoine with only *ectA*. A positive transformant that had the full length transgene inserted as intended was isolated, and proven to produce the corresponding transcript. However, accumulation of the transgene protein product (EctA) could not be shown, by either SDS-PAGE, or mass spectrometry. Neither could ectoine itself, by HPLC, or salt tolerance experiments. A codon optimized version of the *ectA* gene (*OectA*) was designed and cloned into a transformation vector, but no transformants with the *OectA* gene was isolated. Following is a discussion around elements that influenced the outcome of the results.

4.1 *ectA* transcript levels

*ectA* transcripts did not accumulate to high levels, especially in light exposed algae (figure 3.9). This result is consistent with other reports, where chimeric genes with the *rbcL* 5’ region accumulate transcripts to much lower levels than endogenous *rbcL* transcripts. This is partly attributed to an enhancer, that in the endogenous *rbcL* gene lies between +126 to +170 nucleotides relative to the transcription start site (Klein *et al.*, 1994), i.e., the +34 to +78 nucleotides relative to the translation start codon. This sequence was not present in the *ectA* construct, as the whole coding region of *rbcL* was replaced with that of *ectA*. Moreover, the sequence between +329 to +334 was found to be essential for stabilizing the longevity of *rbcL* transcripts in light (Singh *et al.*, 2001). The half-life of chimeric transcripts with the *rbcL* 5’ region has been shown to drop from 5 hours in the dark, to 20 minutes in the light, and this photo-accelerated degradation of *rbcL* transcripts was found to require nucleotides +21 to +41 relative to the transcription start site (Singh *et al.*, 2001).

Regarding the 3’ region used in chimeric constructs, it seems that this region is less important for transcript accumulation (Barnes *et al.*, 2005). Thus, *ectA* transcript levels are probably determined by the 5’ region used.

Lastly, transcript levels would certainly been higher in a homoplastic transformant for the *ectA* insertion. Considering that the transformant isolated was only about 50 % homoplastic (figure 3.5), a two-fold increase could be expected.

Thus, it can be concluded that low levels of transgenic transcripts observed in the isolated *ectA* transformant is due to the insufficiency of the *rbcL* 5’ region to support high levels of transcript, especially in light conditions; effects that can be attributed to the absence of the additional enhancer and stabilizer regions of the coding regions of *rbcL*, and the presence of a sequence within the *rbcL* 5’ region promoting photo-accelerated degradation of the transcripts.
4.2 EctA protein accumulation

The level of transcripts for important endogenous chloroplast genes (including rbcL) has been shown to have a minor effect on protein accumulation (Hosler et al., 1989), indicating in agreement with other studies that endogenous levels of rbcL gene products (rubisco large subunit) are primarily post-transcriptionally regulated. However, it has been reported that this may not the case for recombinant transgenes, where mRNA levels correlate significantly with protein levels (Barnes et al., 2005). Considering the low mRNA levels observed for ectA (figure 3.9), this would be a major bottleneck for EctA protein accumulation in the transformant.

The non-codon-optimized ectA sequence has many codons that are relatively rare in Chlamydomonas chloroplast DNA (figure 3.14, Appendix 1). This is likely to cause a severe inefficiency in translating the transcript, and is probably a second considerable bottleneck for EctA accumulation. The failure of isolating an OectA transformant (see chapter 4.9) was very unfortunate, as such a transformant should have removed this bottleneck.

Another relevant factor is that protein synthesis in Chlamydomonas chloroplast is at its highest when the algae are growing phototrophically, including the synthesis of endogenous rbcL gene product (Stern, 2009). This is unfavorable considering the fact that ectA transcript levels are at their lowest during light conditions (figure 3.9).

It seems understandable why no EctA protein could be detected by SDS-PAGE, and even with mass spectrometry, considering the low levels of transcript during the light, together with lack of codon optimization. However, the question that arises is how other researchers have been able to detect recombinant proteins expressed in Chlamydomonas chloroplast by using the rbcL 5’ region in their constructs. Wang et al. (2008) reports accumulating a non-optimized human gene product to 0.3 % of total soluble protein in a transformant, and Barnes et al. (2005) detected accumulation of recombinant green fluorescent protein (GFP) from a codon-optimized gfp gene.

When comparing the methods employed by these two reports to that of the ectA project, some differences appear. Probably most important is in the way that protein sample preparation and detection methods are done. Both Barnes et al., and Wang et al., grew their recombinant algae mixotrophically with strong illumination in acetate containing media, until reaching a cell density of $10^7$ cells/mL, which is close to the stationary phase. This high density can probably protect some of the algae from photo-degradation mediated by the rbcL 5’ region by physically blocking light before reaching the cells in the middle of the culture (Franklin et al., 2002) (figure 4.1). Furthermore it they demonstrated that exposing the cells to low intensity light increased the amount of recombinant protein (figure 4.1).
The ectA transformant was still in logarithmic phase of growth when protein samples were harvested. This factor has probably influenced the ability of EctA to accumulate before the protein sample preparation. Moreover, protein sample preparation was done in a rather direct way for the ectA transformant. The mentioned researchers employed additional steps to purify the samples, possibly also influencing the results.

Another difference between the works was that Wang et al. (2008) used the rbcL 3’ region in their construct, however (Barnes et al., 2005) showed recombinant transgene accumulation with both rbcL and psbA 3’ region, indicating that the use of psaB 3’ region in the ectA construct was probably not the reason for the inability to detect EctA protein. Lastly, both Wang et al. and Barnes et al. used immunological detection of their recombinant proteins, which might have been more sensitive than the mass spectrometry assay employed for the detection of EctA.

In conclusion, the low levels of ectA mRNA and the lack of codon optimization imply that EctA would not accumulate to high levels. However, it is possible that growing the transformant in acetate medium to near stationary phase and improving the protein sample preparation (and detection) procedure, would allow protein accumulation and detection.

4.3 EctA stability, activity and toxicity

Even if EctA was produced in the isolated transformant, the recombinant protein might be degraded at an accelerated level compared to endogenous proteins. This is a potential problem that is difficult to overcome by genetic methods. One experiment reported a half-life of 1.5 hours for a recombinant protein expressed in the chloroplast of Chlamydomonas (Surzycki et al., 2009).

Additionally, the activity of EctA might be regulated by factors that are absent in the chloroplast, although this is not likely a significant problem, considering that
researchers have detected accumulation of ectoine in transgenic higher plants (Moghaieb et al., 2011).

There was no indication that EctA was toxic or harmful to transformants, as ectA transformants were readily isolated.

### 4.4 Transformosome

A report on factors effecting expression of recombinant proteins in *Chlamydomonas* (Surzycki et al., 2009), found that there was a large variation in the levels of recombinant protein accumulation in different transformants, bombarded with the same transformation vector (a chloroplast homologous integration vector). The researchers used the term “transformosome” to explain the differences, postulating that the transformation procedure had altered the genetic material of the transformants randomly, in addition to the homologous integration. They were able to show that the half-life of recombinant proteins varied in different transformants, indicating that some proteases was affected in high yielding transformants. Conversely, only one ectA transformant was isolated and further analyzed. By analyzing several different transformants it is possible that different levels of recombinant protein accumulation could be found.

### 4.5 SDS PAGE and mass spectrometry

In protein samples prepared from ectA transformant during light and dark conditions no accumulation of EctA was detected by mass spectrometry (MS) analysis. As discussed previously, the growth conditions of transformants (exponential growth phase without acetate) might have been sub-optimal for accumulation of EctA, and the protein sample preparation procedure could have been improved. Another problem in detecting EctA might have been that the SDS-PAGE gel used for MS was overloaded with sample, and/or that the gel-pieces cut out for analysis were too large (figure 3.12). The method used (overloading, and cutting out large gel-pieces), probably made the ratio of irrelevant protein to EctA very large, making detection of EctA hard by MS. An improved method should have been employed, by loading less, cutting several smaller bands, or a combination of the two. A better protein sample preparation could have made the gel-bands sharper, by removing salts, lipids, nucleic acids, or other irrelevant molecules before SDS-PAGE. This would have increased the chances of detecting EctA.

### 4.6 High pressure liquid chromatography (HPLC)

The HPLC was done by an external lab (Dr. Galinski, Germany), with a lyophilized ectA transformant sample. They reported no detection of ectoine. It should be noted that their procedure typically detects only 20% of total ectoine in a sample (Dr. Galinski, personal communication). Furthermore, their sample
preparation protocol is probably optimized for bacteria, which they usually analyze, so the same protocol might not be very effective for \textit{Chlamydomonas}. Thus, it cannot be concluded that there is no ectoine in the transformant by the HPLC analysis, however if there was any it could not have been very abundant.

4.7 Salt tolerance experiment

The salt tolerance experiment did not indicate an increased NaCl tolerance of the \textit{ectA} transformant compared to wild type \textit{Chlamydomonas} (figure 3.14). Although a likely explanation for the lack salt tolerance in \textit{ectA} transformants is low levels, or absence of ectoine, it is still possible that ectoine alone does not increase the salt tolerance of \textit{Chlamydomonas}. The NaCl might adversely affect \textit{Chlamydomonas} in other ways than through the osmotic effect, e.g. inhibiting proteases that are required to dissolve the cell wall after cell division. Replacing NaCl by a non-ionic osmoticum, e.g. mannitol, could give a different result. In conclusion, the experiment did not indicate any ectoine accumulation in the \textit{ectA} transformant.

4.8 EctA substrate availability

\textit{EctB} catalyzes the initial step of ectoine biosynthesis (three steps in total): L-aspartate-\(\beta\)-semialdehyde \(\rightarrow\) L-2,4-diaminobutyrate. It was speculated that L-2,4-diaminobutyrate, the substrate of EctA, could be present in wild type \textit{Chlamydomonas}, thereby eliminating the need for EctB.

If the EctA substrate is absent in \textit{Chlamydomonas}, it would be detrimental to the engineered expression of ectoine in \textit{Chlamydomonas} transformed with only \textit{ectA}. It would effectively explain why no ectoine accumulation could be detected, neither by HPLC, or salt tolerance experiments, regardless of EctA accumulation levels. It should furthermore be noted that also the terminal step in ectoine biosynthesis, catalyzed by EctC, might not occur, either spontaneously or by unspecific enzymatic activity. This would likewise be detrimental to ectoine accumulation. However, it is difficult to determine whether the EctB reaction, EctC reaction, or both, are absent in \textit{Chlamydomonas}.

It is possible that the lack of ectoine accumulation is caused by lack of the EctC reaction. One factor that does not support this hypothesis however, is that it L-2,4-diaminobutyrate accumulation should confer some level of increased salt tolerance, a feature that was not observed.

In conclusion, considering the lack of evidence for EctA accumulation in the transformant it is difficult to evaluate whether EctA substrate availability is a problem or not. Whether the EctB and EctC reactions are occurring or not can not be evaluated before showing accumulation of EctA. It is also possible that EctA accumulates, but does not have enzymatic activity in the chloroplast.
4.9 Optimized *ectA*

A codon optimized version of the *ectA* gene (*OectA*) was constructed, and cloned into the same transformation vector that was used for the *ectA* transformation. Unfortunately, no transformants for the *OectA* construct were isolated, even with several trials of the transformation procedure. Under the suspicion that something was wrong with the transformation vector two tests were done. First, the *OectA* transformation vector (and the *ectA* vector as a control) were digested with BspEI, and KpnI, which cuts the vector in three places, to confirm that the fragment pattern was the same as *ectA* (figure 4.2). Second, to test whether the *atpB* gene was present in the vector, a PCR was performed, using primers that bind within the *atpB* gene (figure 4.3). Both of these tests indicate that the transformation vector was as intended. Furthermore, the sequencing of the transformation vector (the *OectA* + 5’ and 3’ regions was sequenced), showed that this region was OK.

![Figure 4.2](image1.png)

**Figure 4.2 OectA and ectA transformation vector digested with BspEI and KpnI, and run on an agarose gel.** The figure indicates that the *OectA* transformation vector is of the expected size ~9.2 kb. L = DNA ladder displayed in bp

![Figure 4.3](image2.png)

**Figure 4.3 PCR amplification product form OectA and ectA transformation vector, using primers for atpB amplification, run on an agarose gel.** The figure shows that the *OectA* transformation vector contains the *atpB* gene. The primers amplify only a ~1000 bp region of the *atpB* (full length *atpB*=1500 bp). L = ladder displayed in bp.

A control microprojectile bombardment was performed using a different transformation vector (with the *atpB* gene), but this did not either produce any transformants. Together with the tests done for the *OectA* transformation vector, it looked like the problem was with the transformation procedure. A contaminated transformation vector sample by toxins or otherwise was unlikely, as the
transformation vector used was isolated from different maxi preparations, and purified by ethanol precipitation before bombardment. There could have been a problem with the \textit{atpB}-deletion mutant culture, the chemicals, materials, agarose plates, or the equipment used for microprojectile bombardment. However, the transformation procedure was also performed in a different lab in Spain, which did not yield any transformants either. A puzzling observation is that almost no (except for four) \textit{atpB} only transformants were observed. Usually, most of the transformants after a bombardment are \textit{atpB} only transformants, but with the \textit{OectA} transformations they were usually absent. This raises the possibility that the \textit{atpB} sequence might have been damage during the cloning of \textit{OectA}, e.g. causing a frameshift mutation in \textit{atpB}. However, no conclusion no explanation for the failure of acquiring \textit{OectA} transformants was found.

\textbf{Conclusion}

Ectoine did probably not accumulate in the \textit{ectA} transformant. This is either due to the low levels of EctA accumulation, or the absence of the EctB and/or EctC reactions.

It is concluded that the current chimeric \textit{ectA} gene is insufficient in supporting high levels of ectoine accumulation, probably because of the low transcript levels supported, and the lack of codon optimization. Further work is needed to improve the accumulation of EctA to higher levels.

\textbf{Further Work}

The analysis and work done with the \textit{ectA} transformation into \textit{Chlamydomonas} chloroplast revealed interesting results that can aid the continuing work of developing a \textit{Chlamydomonas} cell line that produces ectoine by chloroplast transformation. Primarily efforts to increase the levels of EctA protein accumulation, in addition to improve detection methods of EctA and ectoine should be considered.

For unknown reasons no transformants with the optimized \textit{ectA} gene (\textit{OectA}) could be isolated. Further work should be done to try to isolate an \textit{OectA} transformant, as lack of optimization could be a major bottleneck for EctA accumulation. Full sequencing of the \textit{OectA} transformation vector could elucidate whether the vector itself was the problem. A control microprojectile bombardment procedure should be done.

Furthermore, the \textit{OectA} sequence could possibly be better optimized by applying a different optimization protocol, than was done for \textit{ectA}. This includes generating a codon usage table from a reference set of highly expressed genes, rather than the total chloroplast codon usage (Surzycki \textit{et al.}, 2009).
It could be considered to use a different 5’ region in future chimeric ectA constructs. One study found that the green fluorescent protein gene with atpA and psbD 5’ regions supported the highest levels of transcript and protein accumulation, compared to rbcL, and psbA 5’ regions. However, another study (Surzycki et al., 2009) isolated a Chalmydomonas chloroplast transformant with the currently highest achieved recombinant protein levels (21 % of total cell protein), with a transgene flanked by psbA 5’ and 3’ region. Chimeric 5’ regions might be designed, fusing elements from various genes into one 5’ region.

Experimental modification of the rbcL 5’ UTR by mutating the sequence presumed responsible for photo-accelerated degradation (Singh et al., 2001) could potentially increase the longevity of chimeric transcripts in light. Furthermore, sequences from the coding region for rbcL that have shown to increase transcript levels (chapter 4.1) (Singh et al., 2001), could be fused to a chimeric ectA construct. However, the both strategies are untested, and the latter might render the EctA recombinant protein non-functional.

Transcript levels might also increase by inserting more than one copy of the chimeric, having multiple gene copies in tandem. The presence of homologus regions could however potentially cause unforeseen problems. Lastly, it has been reported that the presence of endogenous introns in chimeric transgenes increased protein expression up to 400% compared to base levels (Potvin and Zhang, 2010).

A higher chance of isolating a high yielding EctA expressing transformant might be given by analyzing several transformants, applying the “transformosome” concept of Surzycki et al. (2009). Furthermore, isolation of homoplasmic lines would increase transcript levels, if initial homoplasmicity is low.

The methods used for detection of EctA protein expression should be improved. Mass spectrometry (MS) can be an efficient and sensitive method, however the MS experiment done with the ectA transformant was not optimized, and could have been improved in several ways (chapter 4.5). For further work with Chlamydomonas ectA experiments this is an important point to consider. Immunological detection is an alternative method, but ectA antibodies are not readily available. An alternative would be to express EctA with a histidine-tag, to aid isolation and detection.

The salt tolerance experiment could be repeated with a non-ionic osmoticum, such as mannitol. This would eliminate any side effects that high levels of ions might have on Chlamydomonas growth, and better test for increased osmotic resistance.

Co-transformation of the additional ect genes (ectB and ectC) could be done, assuming that the reactions catalyzed by EctB and EctC are not redundant.
Appendix 1 – Graphical codon usage analysis

_Pseudomonas stutzeri (O)ectA vs Chlamydomonas reinhardtii_ chloroplast codon usage, generated by the ”Graphical codon usage analysis” online tool (http://gcua.schoedl.de/) (Fuhrmann et al., 2004). Each bar represents each codon of the _ectA_ gene, before and after optimization. The bar value (relative adaptiveness) represents the average occurrence of the codon in _Chlamydomonas_ chloroplast. Codons with below 10% relative adaptiveness are shown in red, and codons with below 20% relative adaptiveness are shown in grey:

*ectA vs Chlamydomonas reinhardtii* chloroplast:

*OectA vs Chlamydomonas reinhardtii* chloroplast:
Appendix 2 - Mass spectrometry results

For information about sample preparation and mass spectrometry method see “Materials and Methods” (chapter 2.5.2 and 2.5.3). All protein matches with a score below 10 were excluded.

Sample data form dark incubated transformant:

<table>
<thead>
<tr>
<th>Accession</th>
<th>Coverage</th>
<th># Peptides</th>
<th>MW [kDa]</th>
<th>Score</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>P11471</td>
<td>46,94</td>
<td>11</td>
<td>25,9</td>
<td>421,96</td>
<td>Oxygen-evolving enhancer protein 2, chloroplast precursor - Chlamydomonas reinhardtii - [PSBP_CHLRE]</td>
</tr>
<tr>
<td>Q42687</td>
<td>47,03</td>
<td>10</td>
<td>24,0</td>
<td>117,49</td>
<td>ATP synthase delta chain, chloroplast precursor - Chlamydomonas reinhardtii - [ATPD_CHLRE]</td>
</tr>
<tr>
<td>P14273</td>
<td>31,62</td>
<td>5</td>
<td>26,9</td>
<td>115,98</td>
<td>Chlorophyll a-b binding protein of LHClI type I, chloroplast precursor - Chlamydomonas reinhardtii - [CB2_CHLRE]</td>
</tr>
<tr>
<td>P12852</td>
<td>22,61</td>
<td>5</td>
<td>21,8</td>
<td>76,32</td>
<td>Oxygen-evolving enhancer protein 3, chloroplast precursor - Chlamydomonas reinhardtii - [PSBQ_CHLRE]</td>
</tr>
<tr>
<td>P15192</td>
<td>17,33</td>
<td>2</td>
<td>16,1</td>
<td>69,18</td>
<td>Chlorophyll a-b binding protein type 2 member 2 - Pinus sylvestris (Scots pine) - [CB22_PINSY]</td>
</tr>
<tr>
<td>Q39615</td>
<td>27,55</td>
<td>6</td>
<td>21,3</td>
<td>64,00</td>
<td>Photosystem I reaction center subunit II, chloroplast precursor - Chlamydomonas reinhardtii - [PSAD_CHLRE]</td>
</tr>
<tr>
<td>P06541</td>
<td>34,38</td>
<td>8</td>
<td>51,9</td>
<td>50,36</td>
<td>ATP synthase subunit beta - Chlamydomonas reinhardtii - [ATPB_CHLRE]</td>
</tr>
<tr>
<td>P12356</td>
<td>29,52</td>
<td>4</td>
<td>24,0</td>
<td>42,63</td>
<td>Photosystem I reaction center subunit III, chloroplast precursor - Chlamydomonas reinhardtii - [PSAF_CHLRE]</td>
</tr>
<tr>
<td>O04386</td>
<td>27,54</td>
<td>8</td>
<td>49,6</td>
<td>42,10</td>
<td>Tubulin beta chain - Chlamydomonas incerta - [TBB_CHLIN]</td>
</tr>
<tr>
<td>P50362</td>
<td>20,05</td>
<td>5</td>
<td>40,3</td>
<td>41,25</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase A, chloroplast precursor - Chlamydomonas reinhardtii - [G3PA_CHLRE]</td>
</tr>
<tr>
<td>P23489</td>
<td>16,42</td>
<td>4</td>
<td>45,0</td>
<td>41,22</td>
<td>Ribulose bisphosphate carboxylase/oxygenase activase, chloroplast precursor - Chlamydomonas reinhardtii - [RCA_CHLRE]</td>
</tr>
<tr>
<td>O97484</td>
<td>13,27</td>
<td>1</td>
<td>12,9</td>
<td>34,64</td>
<td>Histone H2B - Euplotes crassus - [H2B_EUPCR]</td>
</tr>
<tr>
<td>P37255</td>
<td>8,07</td>
<td>5</td>
<td>56,1</td>
<td>33,14</td>
<td>Photosystem II P680 chlorophyll A apoprotein - Chlamydomonas reinhardtii - [PSBB_CHLRE]</td>
</tr>
<tr>
<td>P12853</td>
<td>19,24</td>
<td>3</td>
<td>30,5</td>
<td>32,28</td>
<td>Oxygen-evolving enhancer protein 1, chloroplast precursor - Chlamydomonas reinhardtii - [PSBO_CHLRE]</td>
</tr>
<tr>
<td>O48649</td>
<td>29,28</td>
<td>4</td>
<td>20,6</td>
<td>31,54</td>
<td>ADP-ribosylation factor 1 - Salix bakko (Japanese willow) - [ARF1_SALBA]</td>
</tr>
<tr>
<td>Q43216</td>
<td>11,03</td>
<td>1</td>
<td>15,0</td>
<td>31,12</td>
<td>Histone H2B.5 - Triticum aestivum (Wheat) - [H2BS_WHEAT]</td>
</tr>
<tr>
<td>Q9LLC6</td>
<td>17,68</td>
<td>2</td>
<td>20,2</td>
<td>29,24</td>
<td>Cytochrome b6-f complex subunit petO, chloroplast precursor - Chlamydomonas reinhardtii - [PETO_CHLRE]</td>
</tr>
<tr>
<td>P68688</td>
<td>18,82</td>
<td>2</td>
<td>9,7</td>
<td>28,79</td>
<td>Glutaredoxin-1 - Escherichia coli strain K12 - [GLRX1_ECOLI]</td>
</tr>
<tr>
<td>Q8HTL1</td>
<td>28,49</td>
<td>3</td>
<td>20,2</td>
<td>27,17</td>
<td>Chloroplast 50S ribosomal protein L5 - Chlamydomonas reinhardtii - [RKS_CHLRE]</td>
</tr>
<tr>
<td>Q8HTL5</td>
<td>26,86</td>
<td>3</td>
<td>20,1</td>
<td>24,55</td>
<td>ATP synthase B chain - Chlamydomonas reinhardtii - [ATPF_CHLRE]</td>
</tr>
<tr>
<td>P04352</td>
<td>42,94</td>
<td>3</td>
<td>18,3</td>
<td>24,54</td>
<td>Calmodulin - Chlamydomonas reinhardtii - [CALM_CHLRE]</td>
</tr>
<tr>
<td>P0AA27</td>
<td>39,45</td>
<td>3</td>
<td>11,8</td>
<td>24,45</td>
<td>Thioredoxin-1 - Escherichia coli O157:H7 - [THIO_ECO57]</td>
</tr>
<tr>
<td>P05434</td>
<td>59,17</td>
<td>5</td>
<td>19,4</td>
<td>23,69</td>
<td>Caltractin - Chlamydomonas reinhardtii - [CATR_CHLRE]</td>
</tr>
<tr>
<td>P49202</td>
<td>24,18</td>
<td>4</td>
<td>17,4</td>
<td>22,78</td>
<td>40S ribosomal protein S18 - Chlamydomonas reinhardtii - [RS18_CHLRE]</td>
</tr>
<tr>
<td>O20029</td>
<td>25,65</td>
<td>2</td>
<td>21,0</td>
<td>22,02</td>
<td>Chloroplast 30S ribosomal protein S9 - Chlamydomonas reinhardtii - [RR9_CHLRE]</td>
</tr>
<tr>
<td>Q00471</td>
<td>21,40</td>
<td>3</td>
<td>24,1</td>
<td>21,67</td>
<td>Cytochrome b6 - Chlamydomonas reinhardtii - [CYB6_CHLRE]</td>
</tr>
<tr>
<td>P10898</td>
<td>13,88</td>
<td>4</td>
<td>50,6</td>
<td>19,59</td>
<td>Photosystem I 44 kDa reaction center protein - Chlamydomonas reinhardtii - [PSBC_CHLRE]</td>
</tr>
<tr>
<td>Q9N2N6</td>
<td>10,59</td>
<td>3</td>
<td>49,8</td>
<td>17,30</td>
<td>Tubulin beta chain - Euplotes focardii - [TBB_EUPFO]</td>
</tr>
<tr>
<td>P49728</td>
<td>27,67</td>
<td>2</td>
<td>21,5</td>
<td>16,52</td>
<td>Cytochrome b6-f complex iron-sulfur subunit, chloroplast precursor - Chlamydomonas reinhardtii - [UCR1A_CHLRE]</td>
</tr>
<tr>
<td>P26526</td>
<td>6,69</td>
<td>2</td>
<td>54,7</td>
<td>15,57</td>
<td>ATP synthase subunit alpha - Chlamydomonas reinhardtii - [ATPA_CHLRE]</td>
</tr>
<tr>
<td>O65731</td>
<td>7,11</td>
<td>2</td>
<td>22,0</td>
<td>15,38</td>
<td>40S ribosomal protein S5 - Cicer arietinum (Chickpea) (Garbanzo) - [RSS_CICAR]</td>
</tr>
</tbody>
</table>
Sample data from light exposed transformant:

<table>
<thead>
<tr>
<th>Accession</th>
<th>Coverage</th>
<th># Peptides</th>
<th>MW (kDa)</th>
<th>Score</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>P11471</td>
<td>58,37</td>
<td>14</td>
<td>25,9</td>
<td>620,86</td>
<td>Oxygen-evolving enhancer protein 2, chloroplast precursor - Chlamydomonas reinhardtii - [PSBP_CHLRE]</td>
</tr>
<tr>
<td>P15192</td>
<td>17,33</td>
<td>2</td>
<td>16,1</td>
<td>246,75</td>
<td>Chlorophyll a-b binding protein type 2 member 2 - Pinus sylvestris (Scots pine) - [CB22_PINSY]</td>
</tr>
<tr>
<td>P14273</td>
<td>45,45</td>
<td>6</td>
<td>26,9</td>
<td>149,34</td>
<td>Chlorophyll a-b binding protein of LHCU type I, chloroplast precursor - Chlamydomonas reinhardtii - [CB2_CHLRE]</td>
</tr>
<tr>
<td>Q42687</td>
<td>48,40</td>
<td>11</td>
<td>24,0</td>
<td>114,80</td>
<td>ATP synthase delta chain, chloroplast precursor - Chlamydomonas reinhardtii - [ATPD_CHLRE]</td>
</tr>
<tr>
<td>P06541</td>
<td>22,50</td>
<td>7</td>
<td>51,9</td>
<td>61,04</td>
<td>ATP synthase subunit beta - Chlamydomonas reinhardtii - [ATPB_CHLRE]</td>
</tr>
<tr>
<td>P04264</td>
<td>16,30</td>
<td>7</td>
<td>66,0</td>
<td>49,19</td>
<td>Keratin, type II cytoskeletal 1 - Homo sapiens (Human) - [K2C1_HUMAN]</td>
</tr>
<tr>
<td>O20029</td>
<td>31,94</td>
<td>4</td>
<td>21,0</td>
<td>44,35</td>
<td>Chloroplast 30S ribosomal protein S9 - Chlamydomonas reinhardtii - [R93_CHLRE]</td>
</tr>
<tr>
<td>P23489</td>
<td>28,92</td>
<td>7</td>
<td>45,0</td>
<td>44,35</td>
<td>Ribulose bisphosphate carboxylase/oxygenase activase, chloroplast precursor - Chlamydomonas reinhardtii - [RCA_CHLRE]</td>
</tr>
<tr>
<td>Q39615</td>
<td>15,82</td>
<td>3</td>
<td>21,3</td>
<td>44,29</td>
<td>Photosystem I reaction center subunit II, chloroplast precursor - Chlamydomonas reinhardtii - [PSA_CHLRE]</td>
</tr>
<tr>
<td>P12853</td>
<td>33,33</td>
<td>6</td>
<td>30,5</td>
<td>41,60</td>
<td>Oxygen-evolving enhancer protein 1, chloroplast precursor - Chlamydomonas reinhardtii - [PSBO_CHLRE]</td>
</tr>
<tr>
<td>P26526</td>
<td>16,93</td>
<td>5</td>
<td>54,7</td>
<td>37,10</td>
<td>ATP synthase subunit alpha - Chlamydomonas reinhardtii - [ATPA_CHLRE]</td>
</tr>
<tr>
<td>P50362</td>
<td>18,18</td>
<td>5</td>
<td>40,3</td>
<td>34,78</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase A, chloroplast precursor - Chlamydomonas reinhardtii - [G3PA_CHLRE]</td>
</tr>
<tr>
<td>P12356</td>
<td>29,52</td>
<td>4</td>
<td>24,0</td>
<td>34,27</td>
<td>Photosystem I reaction center subunit III, chloroplast precursor - Chlamydomonas reinhardtii - [PSAF_CHLRE]</td>
</tr>
<tr>
<td>Q93WD2</td>
<td>20,36</td>
<td>3</td>
<td>29,9</td>
<td>29,98</td>
<td>Chlorophyll a-b binding protein CP29 - Chlamydomonas reinhardtii - [CB29_CHLRE]</td>
</tr>
<tr>
<td>O97484</td>
<td>13,27</td>
<td>1</td>
<td>12,9</td>
<td>27,47</td>
<td>Histone H2B - Euplotes crassus - [H2B_EUPCR]</td>
</tr>
<tr>
<td>Q91W63</td>
<td>8,21</td>
<td>1</td>
<td>22,3</td>
<td>27,17</td>
<td>Nicotinamide riboside kinase 1 - Mus musculus (Mouse) - [NRK1_MOUSE]</td>
</tr>
<tr>
<td>Q8H7L1</td>
<td>29,05</td>
<td>4</td>
<td>20,2</td>
<td>26,87</td>
<td>Chloroplast 50S ribosomal protein L5 - Chlamydomonas reinhardtii - [RK5_CHLRE]</td>
</tr>
<tr>
<td>P12852</td>
<td>28,14</td>
<td>5</td>
<td>21,8</td>
<td>25,68</td>
<td>Oxygen-evolving enhancer protein 3, chloroplast precursor - Chlamydomonas reinhardtii - [PSBQ_CHLRE]</td>
</tr>
<tr>
<td>P42380</td>
<td>10,88</td>
<td>3</td>
<td>59,5</td>
<td>23,49</td>
<td>ATP-dependent Clp protease proteolytic subunit - Chlamydomonas reinhardtii - [CLPP_CHLRE]</td>
</tr>
<tr>
<td>Q43216</td>
<td>11,03</td>
<td>1</td>
<td>15,0</td>
<td>23,46</td>
<td>Histone H2B.5 - Triticum aestivum (Wheat) - [H2BS_WHEAT]</td>
</tr>
<tr>
<td>P19824</td>
<td>14,13</td>
<td>3</td>
<td>41,9</td>
<td>22,88</td>
<td>Phosphoribulokinase, chloroplast precursor - Chlamydomonas reinhardtii - [KPPR_CHLRE]</td>
</tr>
<tr>
<td>P49728</td>
<td>27,67</td>
<td>2</td>
<td>21,5</td>
<td>21,48</td>
<td>Cytochrome b6-f complex iron-sulfur subunit, chloroplast precursor - Chlamydomonas reinhardtii - [UCRIA_CHLRE]</td>
</tr>
<tr>
<td>Accession</td>
<td>ID</td>
<td>M</td>
<td>W</td>
<td>L</td>
<td>Description</td>
</tr>
<tr>
<td>-----------</td>
<td>----</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>-------------</td>
</tr>
<tr>
<td>O04386</td>
<td>15,12</td>
<td>5</td>
<td>49,6</td>
<td>21,42</td>
<td>Tubulin beta chain - Chlamydomonas incerta - [TBB_CHLIN]</td>
</tr>
<tr>
<td>O48649</td>
<td>23,76</td>
<td>3</td>
<td>20,6</td>
<td>21,24</td>
<td>ADP-ribosylation factor 1 - Salix bakko (Japanese willow) - [ARF1_SALBA]</td>
</tr>
<tr>
<td>P46284</td>
<td>9,25</td>
<td>3</td>
<td>41,7</td>
<td>20,60</td>
<td>Sedoheptulose-1,7-bisphosphatase, chloroplast precursor - Chlamydomonas reinhardtii - [S17P_CHLRE]</td>
</tr>
<tr>
<td>P37255</td>
<td>8,07</td>
<td>3</td>
<td>56,1</td>
<td>19,88</td>
<td>Photosystem II P680 chlorophyll A apoprotein - Chlamydomonas reinhardtii - [PSBB_CHLRE]</td>
</tr>
<tr>
<td>P48267</td>
<td>12,50</td>
<td>2</td>
<td>19,1</td>
<td>18,78</td>
<td>Chloroplast 30S ribosomal protein S7 - Chlamydomonas reinhardtii - [RR7_CHLRE]</td>
</tr>
<tr>
<td>P35227</td>
<td>10,59</td>
<td>3</td>
<td>62,1</td>
<td>18,60</td>
<td>Keratin, type I cytoskeletal 9 - Homo sapiens (Human) - [K1C9_HUMAN]</td>
</tr>
<tr>
<td>P22274</td>
<td>22,35</td>
<td>3</td>
<td>20,2</td>
<td>18,21</td>
<td>ADP-ribosylation factor - Candida albicans (Yeast) - [ARF_CANAL]</td>
</tr>
<tr>
<td>Q9LLC6</td>
<td>10,10</td>
<td>1</td>
<td>20,2</td>
<td>16,89</td>
<td>Cytochrome b6-f complex subunit petO, chloroplast precursor - Chlamydomonas reinhardtii - [PETO_CHLRE]</td>
</tr>
<tr>
<td>P36863</td>
<td>19,25</td>
<td>3</td>
<td>23,7</td>
<td>15,59</td>
<td>GTP-binding protein yptV4 - Volvox carteri - [YPTV4_VOLCA]</td>
</tr>
<tr>
<td>P35908</td>
<td>3,72</td>
<td>2</td>
<td>65,8</td>
<td>15,43</td>
<td>Keratin, type II cytoskeletal 2 epidermal - Homo sapiens (Human) - [K22E_HUMAN]</td>
</tr>
<tr>
<td>Q00471</td>
<td>15,81</td>
<td>2</td>
<td>24,1</td>
<td>15,30</td>
<td>Cytochrome b6 - Chlamydomonas reinhardtii - [CYB6_CHLRE]</td>
</tr>
<tr>
<td>O65731</td>
<td>7,11</td>
<td>2</td>
<td>22,0</td>
<td>14,65</td>
<td>40S ribosomal protein S5 - Cicer arietinum (Chickpea) (Garbanzo) - [RS5_CICAR]</td>
</tr>
<tr>
<td>Q39434</td>
<td>6,07</td>
<td>1</td>
<td>23,8</td>
<td>13,64</td>
<td>Ras-related protein Rab2BV - Beta vulgaris (Sugar beet) - [RB2BV_BETVU]</td>
</tr>
<tr>
<td>P26958</td>
<td>4,21</td>
<td>2</td>
<td>52,6</td>
<td>12,80</td>
<td>Ribulose bisphosphate carboxylase large chain precursor - Bryopsis maxima (Green alga) - [RBL_BRYMA]</td>
</tr>
<tr>
<td>A5E057</td>
<td>2,57</td>
<td>1</td>
<td>71,2</td>
<td>12,51</td>
<td>Protein RMD9, mitochondrial precursor - Lodderomyces elongisporus (Yeast) (Saccharomyces elongisporus) - [RMD9_LODEL]</td>
</tr>
<tr>
<td>P05434</td>
<td>24,85</td>
<td>2</td>
<td>19,4</td>
<td>12,42</td>
<td>Caltractin - Chlamydomonas reinhardtii - [CATR_CHLRE]</td>
</tr>
<tr>
<td>O63075</td>
<td>7,56</td>
<td>1</td>
<td>26,2</td>
<td>12,19</td>
<td>Chloroplast ATP synthase a chain precursor - Chlamydomonas reinhardtii - [ATPI_CHLRE]</td>
</tr>
<tr>
<td>P38482</td>
<td>5,05</td>
<td>2</td>
<td>61,8</td>
<td>12,10</td>
<td>ATP synthase subunit beta, mitochondrial precursor - Chlamydomonas reinhardtii - [ATPBM_CHLRE]</td>
</tr>
<tr>
<td>P07753</td>
<td>8,81</td>
<td>2</td>
<td>39,0</td>
<td>11,39</td>
<td>Photosystem Q(B) protein - Chlamydomonas reinhardtii - [PSBA_CHLRE]</td>
</tr>
<tr>
<td>P04352</td>
<td>20,86</td>
<td>2</td>
<td>18,3</td>
<td>10,70</td>
<td>Calmodulin - Chlamydomonas reinhardtii - [CALM_CHLRE]</td>
</tr>
<tr>
<td>P10898</td>
<td>7,38</td>
<td>2</td>
<td>50,6</td>
<td>10,17</td>
<td>Photosystem II 44 kDa reaction center protein - Chlamydomonas reinhardtii - [PSBC_CHLRE]</td>
</tr>
<tr>
<td>Q8HTL5</td>
<td>18,29</td>
<td>2</td>
<td>20,1</td>
<td>10,02</td>
<td>ATP synthase B chain - Chlamydomonas reinhardtii - [ATPF_CHLRE]</td>
</tr>
</tbody>
</table>
Appendix 3 – Transformation vector sequences

The following sequence of the ectA transformation vector (pCrc_32_ectA) was compiled based on the sources as follows:

SK+157 with Eco47 restriction site derived sequences are compiled based on information from the article (Salvador et al., 2011). PCrc32 derived sequences are compiled based on information from the article (Blowers et al., 1993). pUC8 sequence was found on “lablife.org” (https://www.lablife.org/g?a=seqa&id=vdb_g2.INMLL2zuPbe7EoyGBg90LiTG1oM-_sequence_bdd831b4eb143227be8de16c9d850df0368f7bb_10). Endogenous chlamydomonas reinhardtii sequences used were found in the published chlamydomonas reinhardtii chloroplast sequence (Merchant et al., 2007). The ectA sequence is as was sequenced.

(Note that the color codes are not the same as in the result figures)

$rbcL$ 5’ region = light green (underlined shows transcribed part)
$ectA$ coding sequence = brown
$psaB$ 3’ region = orange
$pUC$ 8 sequence = blue
BamHI-EcoRI fragment = purple (containing $atpB$ in red).
Sequences leftover from cloning = black

OectA transformation vector (pCrc_32_OectA) (only first part, rest is same as pCrc_32_ectA):

TGATTAAGACACACATAAAAATTTTGCTAGCTTACATTATTTTTATTTCTAAATATATATATAATTTAAAA
TGATTATTTAACACACAAATTATTTAAATTATTATTCCGGACAGTTATTTTTAGGATCGTCACAAG
GAAGTTACATTATTTATCATTATGCTACATTAAACGTATATTAAACACACACCAGTTTT
GTTTTAAGTTTCACACACAGTAAATGTTACGTGTCCTGACACAGACGACGTTACAACCTACATCA
ATTAGTTGGCTCTGTTCAACCATTAGTACAAATTCTAGATTGCTACATTAAATGTCAGAT
TTGCTGACACACAGCTATTGCTGCAAGAAAACGCTCAAGGTGAAATTAGTTGGTTCTATTACAGTTA
CCGTCACCTTCACGTCACATAGATTTATCTGGTTGCAAGTTGCTGTTTGAAGTTCAGTTCAATGCGTG
GTCAAGGTATAGTTTTACGTAATGTTTACTGCAATACAGCTCGTGTTGCTCTGTAATACGGTTGTC
GTTACATGAAACACAAAAATTCACCCACAGACACGACGCTCTCAACAGTTTTATTTCCACACACTTCGCTGTAAC
CAGAAAGACGAAATTTTATCCCAAGTTTTGCATTCAACATTTTCACATAGTAAGAAGAATTAAG
AGAACACGCAATGATCTAGACGGGCCAGGTCGTTTTCGTTATGTAATGTAACATTTTAATTTACTACCTC
AT
Appendix 4 – Solutions and recipes

**E. COLI RELATED:**

**TEG (1 L):**
25 mM Tris-HCl, pH 8.0, 10 mM Na$_2$-EDTA, 50 mM glucose

**Potassium acetate (5M potassium, 3M acetate):**
294.42 g potassium acetate in 100 mL dH$_2$O. Add glacial acetic acid until a pH of 4.6 is reached (about 40-50% of final volume). Bring to 1 liter.

**Lysogeny broth (LB) (1 Liter):**
10 g tryptone, 5 g yeast extract, 10 g NaCl, 200 µL 5 M NaOH. Add dH$_2$O water to 1 liter.

**Lysogeny broth (LB) with 1.5 % agar (1 Liter):**
10 g tryptone, 5 g yeast extract, 10 g NaCl, 15 g agar, 200 µL 5 M NaOH. Add dH$_2$O water to 1 liter. The solution is autoclaved at 121 ° C for 20 minutes.

**Ampicillin 60mg/ml (10 mL):**
Measure 600 mg of ampicillin, and add dH2O to 10 mL. The solution is sterilized by filtering, 1 mL at a time, through a 0.22 µm filters into 10 separate sterile 1.5 mL microfuge tubes. The tubes are stored at -20 ° C.

**Tris-acetate EDTA buffer (TAE) 50x (1 L):**
242 g Tris base, 57.1 ml glacial acetic acid, 100 mL 0.5 M EDTA pH 8.0. Add dH$_2$O to 1 L.

**Agarose gel loading buffer:**
0.25 % bromphenol blue, 0.25% xylene cyanol FF, 30 % glycerol.

**1 % agarose (60 mL):**
0.6 g agarose is added to 60 mL of 1 x TAE buffer. Solution is covered with plastic foil, and brought to a boil in a microwave oven. Cool down to 50 ° C with running water before pouring into tray.

**CHLAMYDOMONAS RELATED:**

**Salt stock 50x (500 ml):**
12.50g NH$_4$Cl, 0.5 g MgSO$_4$ x 7 H$_2$O, 0.25 g CaCl$_2$ x 2 H$_2$O. Add dH2O to 500 mL.

**Phosphate stock 50x (500ml):**
47 g K$_2$HPO$_4$ x 3 H$_2$O (36 g water free), 18 g KH$_2$PO$_4$. Add dH$_2$O to 500 mL.
Hutner trace elements:

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na₂EDTA· 2H₂O</td>
<td>5 g</td>
</tr>
<tr>
<td>ZnSO₄· 7H₂O</td>
<td>2.2 g</td>
</tr>
<tr>
<td>H₃BO₃</td>
<td>1.14 g</td>
</tr>
<tr>
<td>MnCl₂· 4H₂O</td>
<td>506 mg</td>
</tr>
<tr>
<td>FeSO₄· 7H₂O</td>
<td>499 mg</td>
</tr>
<tr>
<td>CoCl₂· 6H₂O</td>
<td>161 mg</td>
</tr>
<tr>
<td>CuSO₄· 5H₂O</td>
<td>157 mg</td>
</tr>
<tr>
<td>(NH₄)₆Mo₇O₂₄· 4H₂O</td>
<td>110 mg</td>
</tr>
<tr>
<td>Distilled water</td>
<td>100 mL</td>
</tr>
</tbody>
</table>

Adjust the pH to 6.5-6.8 with KOH (~1.6 g) at 70°C. Store the solution in a refrigerator (5°C). The solution should have turned to violet color before use. This process takes a while and is necessary.

**High salt (HS) medium (1 L):**
20 ml salt stock [50x], 20 ml phosphate stock [50x], 1 ml trace elements. Add dH₂O to 1 L, and autoclave for 20 minutes at 121°C.

**High salt high acetate (HSHA) (1 L):**
20 ml salt stock [50x], 20 ml phosphate stock [50x], 1 ml trace elements, 2.5 g potassium acetate. Add dH₂O to 1 L, and autoclave for 20 minutes at 121°C.

**DNA extraction buffer:**
[100 mM Tris pH 8.0, 50 mM Na₂-EDTA, 0.5 M NaCl, 10 mM β-mercaptoethanol].

**BLOTTING RELATED:**

**20X SSC saline-sodium citrate:**
NaCl [3M], trisodium citrate [300 mM, pH 7.0 with HCl].

**Labeling buffer:**
Make the following solutions:
(1) Mix in a 1.5 µL microfuge tube 625 µL tris [1 M pH 8.0], 62.5 µL MgCl₂ [1 M], 8.7 µL β-mercaptoethanol [14.4 M], and 303.8 µL sterile dH₂O. This gives a final solution of Tris-HCl [0.625 M pH 8.0], MgCl₂ [62.5 mM], β-mercaptoethanol [125 mM].
(2) 2.5 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES).
(3) Random hexanucleotide primers (pd[N]₆): 50 units/mL in TE [10 mM Tris, 1 mM Na₂-EDTA, pH 7.5]
To make the finished labeling buffer mix 475 µL of solution 1, 500 µL of solution 2, and 25 µL of solution 3.
HYBRIDIZATION:

Na-phosphate buffer [1 M pH 7.2]:
To 134 g Na₂HPO₄ x 7 H₂O (or 89 g Na₂HPO₄ x 2 H₂O, or 71 g Na₂HPO₄) and 4 ml H₃PO₄ [85 %] add dH₂O to 1 L.

Hybridization buffer (100 mL):
Mix 50 mL Na-phosphate buffer [1 M pH 7.2], 200 µL EDTA [0.5 M pH 8.9], 1 g BSA, 7 g SDS, and add dH₂O to 100 mL. Dissolve at room temperature. Takes a long time.

Wash buffer #1 (1 L):
Mix 40 mL Na-phosphate buffer [1 M pH 7.2], 2 mL EDTA [0.5 M pH 8.9], 5 g BSA, 50 g SDS, and add dH₂O to 1 L.

Wash buffer #2 (4 L):
Mix 160 mL Na-phosphate buffer [1 M pH 7.2], 8 mL EDTA [0.5 M pH 8.9], 40 g SDS, and add dH₂O to 4 L.

NORTHERN:

RNA gel loading buffer:
Mix 0.5 mL glycerol [100 %], 4 µL Na₂-EDTA [250 mM pH 8.0] , 2.5 mg Bromphenolblue, 2.5 mg Xylene cyanol FF, and add sterile dH₂O to 1 mL. Treat with DEPC before use.

MOPS buffer [5x]
MgSO4 [10 mM], MOPS [0.5] M, NaCl [2.5 M]. Adjust pH to 7.5 with NaOH. Filter to sterilize, store in the dark.

SOUTHERN:

DNA gel loading buffer:
4 % sucrose, 0.25 % bromphenol blue.

SDS-PAGE:

3X SDS-PAGE sample buffer:
Tris-HCl [188 mM, pH 6.8], SDS [6 %], glycerol [30 %], bromphenol blue [0.03%], β-mercaptoethanol [15 %]

Solution A:
29.2 g acrylamide, 0.8 g N’, N’-bis-methylene-acylamide. Make to 100 mL with
dH$_2$O. Store in refrigerator, in the dark.

**Solution B:**
Dissolve 18.15 g tris base in 60 mL dH$_2$O. Adjust pH to 8.8 with HCl. Add 4 ml SDS [10 %] and 200 µL TEMED (= N, N', N' – Tetramethylenediamine). Make to 100 mL with dH$_2$O and store at room temperature.

**Solution B':**
Dissolve 6.17 g tris base in 60 mL dH$_2$O. Adjust pH to 6.8 with HCl. Add 4 ml SDS [10 %] and 800 µL TEMED (= N, N, N’, N’ – Tetramethylenediamine). Make to 100 mL with dH$_2$O and store at room temperature.

**10X Electrode running buffer:**
30 g Tris base, 146 g glycin, 10 g SDS. Make to 1000 mL dH$_2$O.

**Gel staining solution:**
Coomassie blue R-250 [0.1%], Methanol [46%], acetic acid [8%]

**Destaining solution:**
Methanol [20%], Acetic acid [5%]
Reference list


Louis, P. and E. A. Galinski (1997). "Characterization of genes for the biosynthesis of the compatible solute ectoine from *Marinococcus halophilus* and osmoregulated expression in *Escherichia coli*." *Microbiology* 143 (Pt 4) 1141-1149.


