



Master Thesis

**Development of a vector construct
for the transformation of the
coccolithophore *Emiliana huxleyi***

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*With courage greater than your fear,
jump into the unknown and you will fly!*

a valuable gift from Michi Ware, Buffalo 1996

Abstract

Genetic transformation of eukaryotic cells is a powerful tool to get an insight into gene functions of the studied organisms. The cosmopolitan coccolithophore *Emiliana huxleyi* is an important contributor to climate regulation and therefore a significant object to study. In this work, a transformation vector for the transformation of *E. huxleyi* was designed. It contains a putative promoter region of an endogenous *fcp* gene amplified from genomic DNA, and the resistance gene, *neo*, amplified from a commercially available plasmid, expressing resistance against the antibiotic G418. These two fragments were integrated into the MCS of the basic vector pUC18 creating the novel transformation vector PnpUC of which one clone was used for preliminary transformation experiments. A PDS1000/He microparticle bombardment system served for the delivery of the DNA into the cells. Conducted PCRs of isolated genomic DNA from bombarded cultures that were kept under selective conditions showed dissimilarities compared to genomic DNA from untreated *E. huxleyi* cultures. Investigations of the PCR revealing differences between the WT and modified cultures remain pending.

Keywords: *Emiliana huxleyi*, genetic transformation, *fcp* promoter, *neo* resistance gene, microparticle bombardment,

Declaration

I hereby certify that this thesis has been composed by me and is based on my own work, unless stated otherwise. Material from the published or unpublished work of others, which is referred to in the thesis, is credited to the author in the text. This work has not been submitted for any other degree.

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Symbols and Abbreviations

2N	diploid
°C	degree celcius
Ω	ohm (electrical resistance)
μ	growth rate
μL	microlitre
A	adenine
<i>ad</i>	fill up to
ANT-F/2	Antarctic seawater supplemented with half strengthened Guillard's f-solution
approx.	approximately
bp	basepair
BLAST	basic local alignment search tool
BSA	bovine serum albumin
C	Cytosine
CCMP	culture collection for marine phytoplankton
d	day
DMS	dimethyl sulphide
DMSO	dimethylsulphoxide
DMSP	dimethyl sulphonio propionate
dNTP	deoxy nucleotide triphosphate
DNA	deoxyribonucleic acid
ds	double stranded
<i>e.g.</i>	exempli gratiā (for example)
<i>egfp</i>	gene coding for enhanced green fluorescent protein
EST	expressed sequence tag
<i>et al.</i>	et alii/aliae (and others)
f	femto
μF	micro Farad (unit for electrical capacitance)
<i>fcp</i>	gene coding for fucoxanthin, chlorophyll a/c-binding protein
FCP	fucoxanthin, chlorophyll a/c-binding protein
Fig.	figure

g	gram
G	Guanine
<i>gfp</i>	gene coding for green fluorescent protein
GFP	green fluorescent protein
h	hour
He	helium
HSP	heat shock protein
ID	identification number
<i>i.e.</i>	id est (that means)
k	kilo
kb	kilo base pair
V	voltage
L	litre
LB	Luria Bertani broth
ln	natural logarithm
m	metre
M	molar (mols/litre)
MCS	multi cloning site
min	minute
mL	millilitre
mRNA	messenger ribonucleic acid
n	nano
N	number of cells
NCBI	National Centre for Biotechnology Information
<i>neo</i>	gene coding for neomycin phosphotransferase II
OD	optical density
ori	origin of replication
PCR	polymerase chain reaction
rpm	rounds per minute
RT	room temperature
s	second
SEM	scanning electron microscope
siRNA	small interfering RNA
T	Thymine

Tab.	table
X-Gal	5-bromo-4-chloro-3-indolyl- beta-D- galactopyranoside (substrate for β - galactosidase)
WT	wild type <i>i.e.</i> reference strain (CCMP 1516) used in this work

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1. Introduction

Algae play a major part in climate regulation, since they are accountable for the net primary production of ~52,000,000,000 tons of organic carbon per year, which is about half of the total organic carbon produced on earth each year (Field et al., 1998). However, this is not the only reason why algae are of enormous biological importance. They constitute a heterogeneous group of ~40,000 species, describing a life-form, not a systematic unit, which is one reason why a broad spectrum of phenotypes exists in this group. Algae are very diverse, showing different sizes and shapes and they not only occupy all aquatic ecosystems but also occur in almost all other habitats, some of which are extreme (Hallmann, 2007).

Transgenesis in algae is a complex and fast-growing technology and a powerful tool for the manipulation of these organisms. The introduction of genes into a cell by means of genetic transformation enables us to investigate biochemical processes, either to gain knowledge of cellular biochemistry and get insights of metabolic pathways, or to produce a commercially valuable compound (Dunahay *et al.*, 1995). Selectable marker genes, promoters, reporter genes, transformation techniques, and other genetic tools and methods are already available for various algal species and currently, there are ~25 species accessible to genetic transformation (Hallmann, 2007).

The careful selection of an appropriate target organism stands at the beginning of every algal transformation project. The global impact of the chosen organism is portrayed and outlined in this introduction. Furthermore, a number of possible research subjects that could become objective for the application of a functioning transformation system are introduced.

1.1 The global impact of phytoplankton

The climate of the ocean-atmosphere systems is sensitive to variations of the solar constant and the orbital characteristics of the earth. The seas and oceans effect physical atmospheric processes through the global solar radiation budget (reflection) and meridional heat transport (ocean currents, *e.g.* Gulfstream), and through the trace gas composition of the atmosphere (Holligan, 1992).

However, the properties of oceans surface waters and that of the marine atmosphere are modified also by the optical and biochemical properties of marine organisms, in particular, the phytoplankton (Brierley & Kingsford, 2009).

Biological processes such as phytoplankton photosynthesis contribute to the absorption of atmospheric CO₂ in the ocean which lowers the partial pressure of CO₂ in the upper ocean. The absorption of CO₂ from the atmosphere is thereby promoted, which keeps atmospheric CO₂ concentrations significantly lower than they would be if all the phytoplankton in the ocean were to die (Falkowski *et al.*, 2000).

CO₂ is incorporated into organic matter by phytoplankton of which much is rapidly re-oxidized within the euphotic zone. However, a small proportion (~10% of net primary production) is transferred to deep water and the sediments, so that an atmosphere-to-deep water gradient in CO₂ concentration is maintained, which represents the organic carbon pump (Holligan, 1992).

In addition to the organic carbon pump, several phytoplankton and zooplankton species form CaCO₃ shells that sink into the interior of the ocean, where it is partly dissolved and partly stored in the geological archive (Westbroek *et al.*, 1993). This inorganic carbon cycle leads to a reduction in surface ocean dissolved inorganic carbon (DIC) relative to the deep ocean and is therefore sometimes called the “carbonate pump”. However, it can be predicted that the sink strength will almost certainly weaken (Falkowski *et al.*, 2000) due to increasing anthropogenic release of CO₂ in the atmosphere.

Lovelock *et al.* (1972) first suggested, that DMS is the natural sulphur compound that transfers sulphur from the seas through the air to land surfaces and is therefore considerable important in the global sulphur cycle. The major precursor of DMS is dimethylsulphoniopropionate (DMSP), a compatible solute found in various groups of marine algae (Steinke *et al.*, 2002). Enzymatic cleavage by DMSP lyase (dimethylpropiothetin dethiomethylase) is thought to be the major process for DMS production in marine environments. DMSP lyase isozymes have been found in various marine organisms (Wolfe, 2000, Steinke *et al.*, 2002, Steinke *et al.*, 1998). DMS excreted by most species of phytoplankton escapes to the air where it reacts to form a sulphate and methane sulphonate aerosol (Shaw,

1983). These aerosol particles act as cloud-condensation nuclei (CCN) in the marine atmosphere (Charlson *et al.*, 1987).

The term “phytoplankton”, coined in 1897, describes a diverse, polyphyletic group of mostly single-celled photosynthetic organisms that drift with the currents in marine and fresh waters. Although accounting for less than 1% of earth’s photosynthetic biomass, these microscopic organisms are responsible for more than 45% of our planet’s annual net primary production. Whereas on land, photosynthesis is dominated by a single clade (the Embryophyta) containing nearly 275,000 species, there are fewer than ~25,000 morphologically defined forms of phytoplankton; they are distributed among at least eight major divisions or phyla (Falkowski *et al.*, 2004, Field *et al.*, 1998).

1.1.1 The coccolithophore *Emiliana huxleyi*

The major taxonomic groups of phytoplankton, such as diatoms and colonial algae (*e.g. Phaeocystis*), are prevailed by coccolithophores, the dominant calcifying group of phytoplankton (Holligan, 1992). The coccolithophores belong to the division Haptophyta (also known as prymnesiophytes) (Jordan & Green, 1994), a group of biflagellates, generally found in marine habitats, with a yellow-brown pigmentation (Westbroek *et al.*, 1993). Haptophyte cells are usually covered with organic scales which are formed intracellularly. These calcified scales, called "coccoliths", have highly elaborate shapes, and the "coccosphere" surrounding a single cell may harbour types with different morphologies. Coccolithophores are most abundant in the open ocean, where they sometimes outnumber all other types of phytoplankton (Castro *et al.*, 1997). In the present ocean about 150 coccolithophore species are known (Westbroek *et al.*, 1993).

The coccolithophore *Emiliana huxleyi* (Lohmann) Hay and Mohler (Fig. 1) is one of the most abundant and widely distributed photosynthetic unicellular eukaryotes in modern oceans.

E. huxleyi was first described from ocean sediments about 270,000 years old and is thought to have appeared first in the tropics and subsequently spread to higher latitudes (Thierstein *et al.*, 1977). *E. huxleyi* is considered to be the world's major producer of calcite (Westbroek *et al.*, 1985). Not only its coccoliths, but also a

suite of organic biomarkers (long-chain alkenones and alkyl alkenoates) provide a highly characteristic record in the sedimentary archive (Westbroek *et al.*, 1993). It is recognized to be an important factor in determining the exchange of CO₂ between the oceans and the sediments (Dymond & Lyle, 1985). Steinke *et al.* (2002) hypothesise that *E. huxleyi* is the most important producer of DMS in a typical North Atlantic coccolithophore bloom and, hence, would contribute most of the DMSP lyase activity.

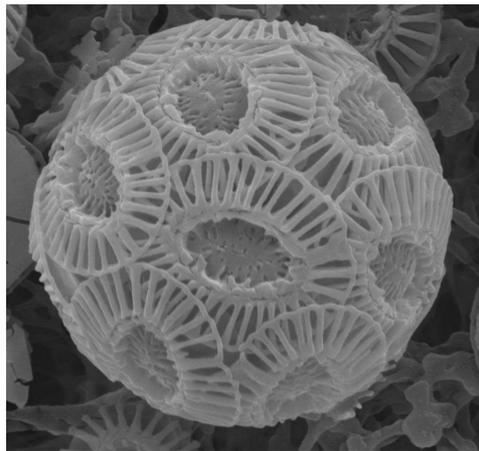


Figure 1: Scanning electron microscope image of the coccolithophore *Emiliana huxleyi* (Langer *et al.*, 2006).

E. huxleyi occurs in all oceans except for the polar waters (Brand, 1994, Winter & Siesser, 1994, Paasche, 2002, Marsh, 2003) and typically accounts for 20-50% of the total coccolithophore community in most oceanic areas (McIntyre & Bé, 1967). With its diameter of 5-10 μm *E. huxleyi* is one of the smaller coccolithophores. At one stage of its life cycle the cell is covered with one or several layers of heterococcoliths, 2-4 μm long and consisting of calcite and macromolecular organic material. Not only these non-motile diploid coccolith-bearing cells (C-cells), but also naked cells (N-cells) and motile, haploid scale-bearing cells (S-cells) participate in the life cycle of this species (Klaveness, 1972, Laguna *et al.*, 2001). N-cells are morphologically very similar to C-cells, but do not calcify (Klaveness & Paasche, 1971). The S-cells possess two cilia and are covered with organic scales formed in the cisternae of the Golgi apparatus (van der Wal *et al.*, 1985).

1.1.2 *Emiliana huxleyi* blooms

The coccolith-producing *E. huxleyi* is known for its formation of extensive ocean blooms with concomitant production of large amounts of DMS. Coccoliths, which readily reflect light, and DMS, which enhances cloud formation, contribute to increased albedo and thus have a cooling influence on the climate (Graham *et al.* 2000). Maximum concentrations of *E. huxleyi* of values as high as $1.2 \cdot 10^7$ cells L^{-1} have been recorded (Berge, 1962). Coccolithophore blooms reach their greatest seasonal mean annual total of $6.3 \cdot 10^5$ km^2 in the subarctic North Atlantic (Westbroek *et al.*, 1993).



Figure 2: *E. huxleyi* summer bloom off the coast of Cornwall.

The reflexion of the sunlight is especially promoted by the detached coccoliths.

(http://www.sanger.ac.uk/Info/Press/gfx/050811_bloom.jpg)

Light scattering by coccoliths represents a special case of biological effects on surface ocean optics, with values for sub-surface reflectance exceeding 30% (Balch *et al.*, 1991) compared to 3-5% in the absence of coccoliths. With the density of coccoliths beyond $3 \cdot 10^5$ mL^{-1} within blooms of *E. huxleyi*, extreme conditions for biological warming and shallowing of the mixed layer are predicted (Kirk, 1988).

Termination of these blooms is accompanied by massive release of organic and inorganic matter to the water column, including detached coccoliths that reflect sunlight and are readily detectable in satellite images (Tyrrell & Merico, 2004) (Fig. 2).

1.1.3 Bloom termination by viruses

Zooplankton grazing, physical wash-out and light or micronutrient limitation are some factors that are responsible for the termination of natural phytoplankton blooms (Westbroek *et al.*, 1993). Many eukaryotic algae, however, are known to be infected by viruses (Hallmann, 2007). *E. huxleyi* from marine nanoplankton samples have been reported to contain viral particles approximately 200 nm in diameter (Manton & Leadbeater, 1974).

Several studies have investigated the role of viruses in controlling the bloom development of *E. huxleyi* (Bratbak *et al.*, 1993, Bratbak *et al.*, 1995, Brussaard *et al.*, 1996, Wilson *et al.*, 1998, Wilson *et al.*, 2002b, Wilson *et al.*, 2002a). These investigations clearly showed that viruses are responsible for the decline of *E. huxleyi* blooms. In some cases, viral lysis could account for 25 to 100% of the net mortality of *E. huxleyi* (Brussaard *et al.*, 1996).

Wilson *et al.* (2002b) isolated two viruses from a dying *E. huxleyi* bloom in the Western English Channel and revealed that they were lytic viruses approximately 170 nm – 190 nm in diameter having an icosahedral symmetry. Phylogenetic analysis places one of these two viruses (EhV-86) in a new genus (Coccolithovirus) within the family Phycodnaviridae (Schroeder *et al.*, 2002). Several genomes of these algae infecting dsDNA viruses have been sequenced (Van Etten *et al.*, 2002).

Regulated programmed cell death processes have been documented in several phytoplankton species and are hypothesized to play a role in population dynamics. The mechanisms leading to the coordinated collapse of phytoplankton blooms are, however, poorly understood (Vardi *et al.*, 2007). Wilson *et al.* (2005) postulated that the sphingolipid biosynthesis pathway (ceramide formation), encoded in the genome of EhV-86, could be implicated in the regulation of apoptosis in infected *E. huxleyi* cells. Therefore, one theory is that this algal virus encodes a mechanism for inducing apoptosis as a strategy for killing the host cell and disseminating progeny virions during the infection cycle (Wilson *et al.*, 2005).

1.2 Reverse genetic tools to manipulate gene expression

The ability to switch certain genes of an organism's genome on or off via reverse genetic tools delivers a valuable tool for the elucidation of certain pathways and allows us to study biochemical processes as well as viral infection mechanisms.

This can be done through knock-out mutants that can be created by the introduction of interfering RNA (RNAi) that is introduced into the cells and acts sequence specifically by silencing genes on the posttranscriptional level. Double-stranded RNA suppresses the expression of a target protein by stimulating the specific degradation of the target mRNA.

The silencing of certain genes by implementation of anti-sense RNA into the host cell can also be achieved using a vector approach. With this strategy, knock-out mutants can be generated by transformation of the host with a vector that expresses anti-sense constructs of the knock-out target genes. In this approach, artificial anti-sense RNA is expressed, leading to complementary sequences to the desired target genes, hence, hybridizing to the target mRNA which prevents it from being translated into protein.

A vector containing a promoter and selection or marker gene in front of a multiple cloning site (MCS) is therefore desired. The gene of interest can then be cloned into the MCS and transformed and expressed in the target organism.

A stable transformation of the microalga *E. huxleyi* would allow the generation of knock-out mutants *e.g.* of genes that are expressed during viral infection. The expression of selected virus genes would make it possible to get an insight into the mechanisms of viral infection, including gene-functions or pathways and processes.

1.2.1 Genetic transformation of microalgae

Genetic transformation is a process by which the genetic material carried by an individual cell is altered by the incorporation of foreign (exogenous) DNA into its genome.

The ability to manipulate microalgae via genetic engineering in order to introduce or optimize desired traits will facilitate more extensive exploitation of these organisms since interest in the use of microalgae for research as well as commercial applications has increased in recent years (Dunahay *et al.*, 1995).

Genetic engineering in several microalgae such as *Chlamydomonas reinhardtii* (Debuchy *et al.*, 1989, Kindle *et al.*, 1989) and the simple multicellular organism, *Volvox carteri* (Schiedlmeier *et al.*, 1994), has been carried out successfully (Kathiresan & Sarada, 2009). Genetic transformation (*Agrobacterium*-mediated, electroporation, biolistic gun, etc.) protocols are being developed and constantly improved for several species such as *C. reinhardtii*, *V. carteri* and *Chlorella*. The full potential of genetic transformation has not been realized for most of the algal species (Travella *et al.*, 2005).

The strongest barrier for foreign DNA to enter cells is the cell membrane that has to be penetrated. Several methods for the introduction of DNA into the nucleus have been reported, including particle bombardment (Debuchy *et al.*, 1989, Kindle *et al.*, 1989, Klein *et al.*, 1987), electroporation (Brown *et al.*, 1991), and agitation with glass beads (Kindle, 1990) or silicon fibers (Dunahay, 1993).

Microparticle bombardment even works for the tough silica cell walls of diatoms and has been performed several times (Dunahay *et al.*, 1995, Apt *et al.*, 1996, Poulsen *et al.*, 2006, Kroth, 2007). It is also recommended as the method of choice for the novel transformation of organisms where the protocol may include a number of uncertain experimental issues (Hallmann, 2007).

For a successful transformation, several prerequisites have to be established. An axenic culture is ideal so that after transformation the weakened culture cannot be overgrown by bacteria. Suitable selective agents or markers that can be expressed by the organism and are effective for the organism have to be defined. A vector containing essential parts, such as a promoter, antibiotic resistance or marker gene and a multiple cloning site, has to be designed and created. For the selection of transformed algae, a method must be established or be available that allows for regeneration of the target species from single cells, ideally on agar plates.

1.3 Aim of this work

E. huxleyi is the most abundant coccolithophore and an important member of the marine phytoplankton, whose bloom collapsing has been frequently linked to virus control in the marine environment (Evans *et al.*, 2009, Kegel *et al.*, 2007). Further studies in order to fully assess the biogeochemical impact of *E. huxleyi* bloom termination by viruses are therefore desired.

The creation of a transformation system for *E. huxleyi* could initiate a series of experiments for the study of viral affliction of the coccolithophore. The mechanisms of DNA introduction into the host cell, the metabolic regulations, especially concerning the ceramide pathways, and induction of metacaspases in the host cell, introducing programmed cell death, could be investigated.

An understanding of the coccolithophore's single metabolic pathways, like the yet unknown regulation of the microalga's coccolith-production, could be facilitated.

The aim of this work is to design a novel vector construct for the transformation of the coccolithophore *E. huxleyi*. This construction has to contain essential components, for successful ongoing studies. An appropriate basic vector (*i.e.* yield of high copy numbers, containing ori. and resistance gene for the selection of bacterial clones) as starting construction has to be defined. The sequence of an endogenous controllable promoter which would serve best for subsequent transformation experiments has to be determined, isolated, amplified and provided with suitable restriction sites for further works. For the identification of positively transformed clones an *E. huxleyi* suitable antibiotic resistance and its gene, or an appropriate marker gene, have to be chosen. These genes also have to be amplified with primers providing restriction sites. The final vector should also contain a multiple cloning site that can eventually serve for the insertion of exogenous genes of interest into the target organism. Additionally, prerequisites such as the growth on solid media for further application of this vector should be implemented.

2. Materials and Methods

There were several tasks to be fulfilled for the conductance of this work. First of all, growth experiments with the coccolithophore *Emiliana huxleyi* in liquid as well as on solid media under stressed and non-stressed conditions were conducted; second, preliminary work like searching for sequences and preparing essential constituents (*e.g.* primerdesign) had to be performed; third, single components of this work had to be amplified and prepared; fourth, several cloning experiments for the final preparation of the single sequences and to build the transformation system had to be executed; and finally at last, the biolistic experiment for the possible transformation of *E. huxleyi* and posttranslational experiments create the end of this thesis.

2.1 Growth experiments

A previous diploma thesis (Strauss, 2008) has shown that the alga *E. huxleyi* is sensitive only against a few antibiotics. Three of the more harmful antibiotics are G418 s, puromycin, and chloramphenicol. Therefore, growth experiments for the verification of the best suited antibiotic and its concentration were performed. In addition, the resistance of *E. huxleyi* to the antibiotic kanamycin and its possible enhancement of growth of the alga was to be verified. As standard condition, all growth experiments - both liquid and solid - were incubated at 15°C and at approximately 150 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ in a 16:8 light:dark cycle.

2.1.1 Antibiotic verification in liquid media

E. huxleyi strain CCMP 1516 (Lohmann, 1902, Hay *et al.*, 1967), obtained from the Plymouth Marine Laboratory (Plymouth, UK) was grown in liquid ANT-F/2 medium (Guillard, 1975) according to the previous work of Jan Strauss (2008). Several conditions following Strauss (2008) were set up in triplicates as shown in Tab. 1.

Table 1: Used antibiotics and their applied concentrations.

Antibiotic	Concentration
Kanamycin	500 – 1000 µg/mL
G418	500 µg/mL
Puromycin	50 µg/mL
Chloramphenicol	100 µg/mL

Each condition contained kanamycin plus the antibiotic stated in Tab. 1. The cultures were inoculated with $10 \cdot 10^3$ cells/mL.

2.1.2 Determination of the growth rate

To determine cell density and size spectrum, the cell count was performed with a Multisizer Coulter Counter (Beckmann Coulter GmbH, Germany).

Culture flasks were gently shaken as to detach settled cells from the bottom and ensure homogenous suspension of the cells before sampling. Cultures with densities above $20 \cdot 10^3$ - $30 \cdot 10^3$ cells/mL were diluted with ANT-F/2 to a volume of 20 mL from which the Coulter Counter used 500 µL for analysis of the probe. Dilution factors from 2-400 were used since the Coulter Counter measures most accurate between $10 \cdot 10^3$ - $20 \cdot 10^3$ cells/mL.

For cultures showing a typical growth curve, the relative growth rate was determined using the following formula:

$$\mu = \frac{\ln N_t - \ln N_0}{t - t_0}$$

with μ = relative growth rate

N_t = cell counts [cells/mL] at time t

N_0 = cell counts [cells/mL] at time 0

t = time t [d]

t_0 = time 0, starting time

(Schlegel, 1992)

2.1.3 Growth on solid media

First attempts of growth experiments on solid media were performed with ANT-F/2 medium and the addition of 1 % Bacto Agar (Becton, Dickinson and Company, USA). The media were autoclaved before adding 1 mg/mL kanamycin and one of the three antibiotics (G418, puromycin, chloramphenicol) using the same concentrations as in liquid media. Enough plates were poured so that three replicates of each condition (untreated, containing only kanamycin, and plates containing kanamycin plus one of the three selective antibiotics) could be examined. These plates were inoculated with 500 μ L culture at a concentration of $10 \cdot 10^3$ cells/mL. For two hours the plates were incubated in upright position for the culture volume to integrate into the agar medium and then turned upside down.

However, as stated in Laguna *et al.* (2001) ANT-F/50 medium was used for further experiments. 1.5 % Bacto Agar was added before, and supplement of nutrients, vitamins and antibiotica after autoclaving. Plates with F/50 medium (containing $1/25$ supplements of F/2 medium) were inoculated with different volumes containing a dilution series of $50 \cdot 10^3$ to $1 \cdot 10^3$ cells in total.

2.2 Preliminary work

Before cloning experiments could start, some basic questions had to be answered. An optimal cloning vector, its availability and the right sequences for a suitable promoter were investigated. The primer design and amplification as well as preparation of the single components for the vector were accomplished.

2.2.1 Selection of a suitable vector

Amongst miscellaneous opportunities, it was decided to select a plain basic vector (such as pUC18/19, pBlueskript, or pGEM-T), containing a suitable promoter, selection gene for bacteria and a multicloning site (MCS) as a start. The basic construct should be modified by cloning an *E. huxleyi*-suitable promoter and marker or selection gene into this vector. For this purpose, the available pUC18 vector (provided by M. Lucassen, Alfred-Wegener Institute, Bremerhaven) was chosen.

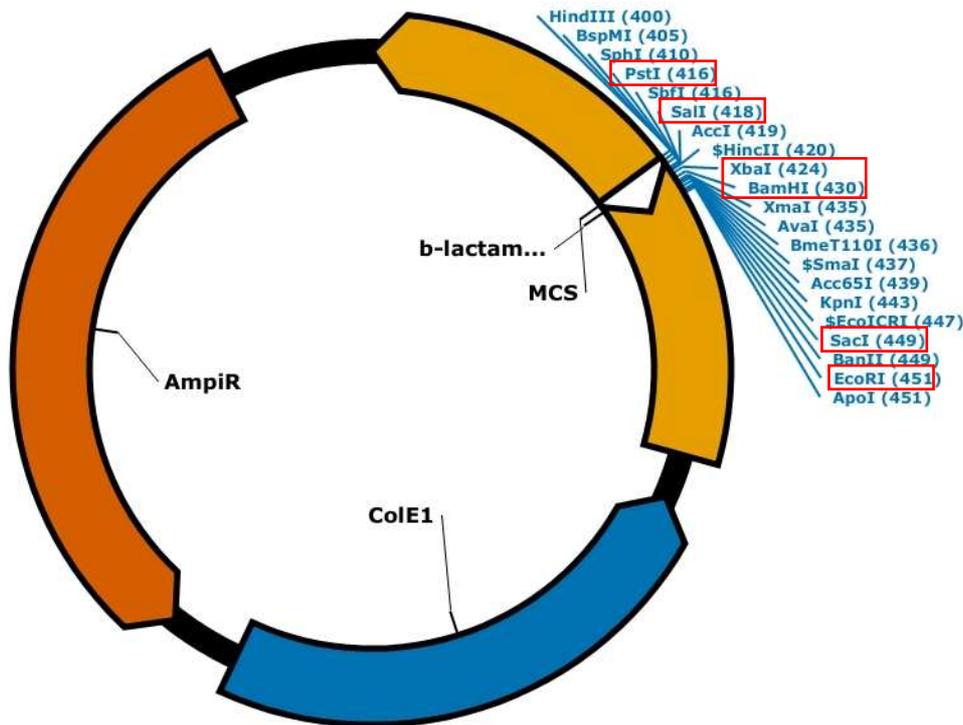


Figure 3: pUC18 vector (2686 bp) as a basic vector to be modified for later transformation of *E. huxleyi*.

The MCS shows restriction sites with red boxes, that are suitable to insert promoter, resistance gene, and marker gene. Resistance against ampicillin (Amp^R encoded by the *bla* gene), the β -galactosidase (b-lactam), and the ori. from ColE1 are indicated.

Fig. 3 shows the pUC18 vector with restriction sites in the MCS and their position in the vector. Enzymes marked with a \$ before their name indicate an enzyme that generates blunt ends which are unsuitable for directional cloning. (ANGERSLOUSTAU, 2007). Enzymes that were selected for the cloning strategy are depicted with red boxes.

The location of the β -galactosidase gene in the MCS facilitates the selection of positively transformed clones by blue-white screening. This works because the amino- and carboxyl domains of β -galactosidase need not be carried on the same molecule to generate β -galactosidase activity. Instead, two inactive fragments of the polypeptide chain, one lacking the amino-terminal region (the α -acceptor) and the other the carboxy-terminal region (the α -donor), are able to associate both in vivo and in vitro to form a tetrameric active enzyme. This unusual form of complementation, called α -complementation, is widely used in molecular cloning to monitor insertion of foreign DNA sequences into vectors encoding the amino-terminal (α -donor) fragment of β -galactosidase (Sambrook & Russell, 2001). In order to be able to perform directional cloning, two restriction enzymes need to be chosen that do not generate the same overhangs. Additionally, they should not cut

the resistance gene, the marker gene, or the promoter, and perform under similar conditions to be able to conduct a double digest, employing both enzymes in one reaction.

2.2.2 Promoter search

A promoter characterizes a sequence situated upstream of a gene that indicates the beginning of a transcription site. This sequence is needed for the RNA-polymerase to recognize the starting point of the transcription of a gene.

Known sequences of FCP (fucoxanthin chlorophyll *a/c*-binding protein), HSP60 and HSP70 proteins (heat shock proteins) of related species such as *Thalassiosira pseudonana* (a diatom) or *Phaeodactylum tricornutum* (another diatom) – or others – were looked up at NCBI. These sequences were blasted (Altschul *et al.*, 1990) against the *E. huxleyi* proteome accessed at the jgi (joint genome institution) homepage (<http://genome.jgi-psf.org/Emihu1/Emihu1.home.html>). Found hits were screened for a “good” E-value (expectation value) which indicates the number of different alignments with scores equivalent to or better than the found hit that are expected to occur in a database search by chance (<http://www.ncbi.nlm.nih.gov/Education/BLASTinfo/glossary2.html>). Meaning, the lower the E-value, the more significant the score – so E-values smaller than E^{-20} were considered.

A similar starting point, compared to closely related species that are shown at the jgi BLAST page, and a definite start of translation (startcodon ATG) in the nucleotide sequence were important. The protein IDs and 500 bp upstream the translation starting point were copied into a separate text-file for the found *fcf*, *hsp60*, and *hsp70* gene sequences respectively and saved. An alignment of these sequences using clustalW was performed (Larkin *et al.*, 2007). Subgroups that show more similarities (homologies) could visually be identified using the program clustalX. It seemed that some patterns occurred in several sequences that were not aligned by the alignment program clustalW. Some subgroups of sequences showing similar patterns were aligned and compared visually (data not shown).

However, since for persistent expression of the selection gene a high expressing promoter is necessary, sequences of high expressed ESTs (Kegel *et al.*, in press) were blasted against the jgi *E. huxleyi* genome database. The found sequences

were compared to the previously inspected putative promoter sequences and the one possessing the protein ID 460117 (EVC02389) was chosen to be amplified.

For the investigation of a termination signal, the same procedure can be followed.

2.2.3 Primer design

Three vectors, containing the resistance genes against G418, puromycin, and chloramphenicol were ordered. These three genes should be amplified out of the vector and then cloned into the pUC18 vector. Using the online source primer3 resulted in primers that did not directly start and end enclosing exclusively the desired sequences. Primer3 results led to too many basepairs between promoter and resistance gene. Primers for the putative promoter sequence, the resistance *neo* (expressing resistance against G418), and the marker gene *egfp* (coding for an enhanced green fluorescence protein), were therefore created manually. It was also attempted to create primers that include desired restriction sites in their sequence which was not always possible. Thus modifications to some primers were done such that the restriction sites of the chosen restriction enzymes were added at the 5' end plus 4 to 5 bp, resulting in the primer sequences stated in the following table:

Table 2: Designed primers containing suitable restriction sites for the amplification of the promoter region, resistance gene *neo*, and marker gene *egfp*.

Sequences in red and italics indicate wanted restriction sites of future utilized restriction enzymes: *EcoRI* for FPrf, *SacI* for FPrr, *BamHI* for G418f, *XbaI* for G418r, *Sall* for GenSalf, and *PstI* for GenPstr. Bold letters designate start and end point of transcription sites. Mismatches of primers with the target sites that had to be taken into account are not underlined.

Name	Sequence	T _m [°C]	Length [bp]
FPrf	5' <u>ACACA</u> <i><u>GAATTC</u></i> TGTGTGGCTTGAG 3'	61.0	24
FPrr	5' TTAAG <i><u>GAGCTC</u></i> GGTGAGGAAGGAG 3'	62.4	23
G418f	5' <u>TATAATA</u> <i><u>GGATCC</u></i> ACTATAGGAGG 3'	57.6	24
G418r	5' <u>AGACAGCGAGCT</u> <i><u>TCTAGA</u></i> TTTAG 3'	58.9	23
GenSalf	5' <u>TATAC</u> <i><u>GTCGAC</u></i> ATGGTGAGCAAGGGCGAGGAG 3'	72.1	32
GenPstr	5' ATACA <i><u>CTGCAGCT</u></i> TTACTTGTACAGCTCGTCCAT <u>GCCG</u> 3'	72.7	38

Results of primer3 also reveal information about possible secondary structures among the primers that could be formed. Since primer3 was not used, this check had to be done in RNAcofold to exclude potential primer dimer, hairpin loops and other secondary structures.

2.2.4 DNA isolation

Genomic DNA from *E. huxleyi* was isolated using DNeasy Plant Mini Kit (Qiagen, Germany). A culture grown to late exponential or steady state phase was allocated into 50 mL tubes, centrifuged for 15 min at 4000 rpm. The supernatant was discarded, the pellet resuspended in 1.5 mL medium, allocated into 2 mL tubes, centrifuged for 5 min at 10000 rpm, and the supernatant was discarded again. Then given instructions in the Qiagen manual were followed. For short term storage the DNA was kept at 4°C or frozen at -20°C for long term storage. Utilized material and equipment is listed in the appendix.

2.2.5 Preparation of backups

The transformation of *Escherichia coli* TOP10 cells is vital for long term storage and for the production of vector from a positive selected clone containing the desired feature. For the preparation of backups, the used material and equipment is listed in the appendix.

2.2.5.1 Generation of electrocompetent cells

To generate purchased vector and also for the further course of this work electrocompetent *E. coli* (TOP10) cells were prepared according to the following protocol.

5 mL LB medium with a concentration of 200 µg/mL streptomycin were incubated over night with *E. coli* TOP10 cells. The over-night culture was completely transferred into a 500 mL Erlenmeyer shaking flask containing prewarmed 200 mL LB medium. The culture was grown to an OD₅₄₀ = 0.5 – 0.7. It was then aliquoted into four prechilled 50 mL tubes and kept on ice for at least 15 min. The tubes were centrifuged under the same conditions in each step

(4000 g, at 2°C, for 15 min.). After centrifugation the pellets were resuspended carefully in 40 mL Washing Buffer each and centrifuged again. The pellets were resuspended in 20 mL Washing Buffer each, joined into two tubes and centrifuged. The pellets were resuspended in 10 mL Washing Buffer, joined into one tube and centrifuged. The pellet was now resuspended in 700 µL Suspension Buffer which was aliquoted in 40-50 µL aliquots into prechilled kryo vials. These were shock frozen in liquid nitrogen, and the electrocompetent cells were stored at -80°C.

2.2.5.2 Transformation of electrocompetent cells

The transformation of electrocompetent cells was performed with a Gene Pulser Xcell Electroporator (BioRad, USA). A variable amount of ligation reaction (0.5 µL up to 2 µL) or vector (0.5 µL) was added to 40-50 µL of electrocompetent *E. coli*, stirred carefully with the pipette tip, and then transferred into the 1 mm quartz electroporation cuvette. The cells were transformed at 1.8 kV, 25 µF, and 200 Ω. 0.5 mL prewarmed LB-medium was quickly added to the cells, and then transferred into a 2 mL tube containing 1 mL LB-medium in total. The cells were allowed to express their newly added feature (*i.e.* antibiotics resistance) while incubating at 37°C for one hour. A variable amount of 40 µL up to 100 µL was spread on an agar-plate that contained 100 µg/mL of the suitable antibiotic (ampicillin). If blue-white screening was planned, 16 µL X-Gal (promega, USA) (+40 µL LB-medium) were spread onto the plate with a Drigalski spatula.

The plates were incubated over night at 37°C. On the next day, 5 mL liquid LB-medium containing ampicillin were inoculated with clones picked from the plates and incubated on a shaker at 37°C over night.

2.2.5.3 Plasmid preparation

Positive selected clones that contain plasmids with inserts were isolated from their host using the QIAprep Spin Miniprep Kit (Qiagen, Germany). The preparation was performed according to the instructions given in the manual.

2.3 PCR reactions for amplification

PCR reactions for the amplification of the single cloning components were performed. Primers (listed in Tab. 2) were generally used at a concentration of 10 μ M for amplification reactions. In the following the single PCR reactions are described. The reagents and equipment that was used for the PCR reactions is listed in the appendix.

2.3.1 Amplification of the promoter

Several conditions were tested to optimize the amplification of the promoter region (Protein ID: 460117) from genomic *E. huxleyi* DNA. The addition of 5% DMSO which prevents sequences from forming secondary structures, and 1 M betaine that also facilitates strand separation was necessary (Frackman *et al.*, 1998). The following PCR setup for a 25 μ L reaction was finally used:

Table 3: Final PCR setup for the amplification of the *E. huxleyi* promoter region from genomic DNA

Substance	Amount
Molecular grade H ₂ O	<i>ad</i> 25.0 μ L
5Prime Mastermix	10.0 μ L
DMSO	1.25 μ L
5M Betain	5.0 μ L
Primer FPrf	1.0 μ L
Primer FPrr	1.0 μ L
Sample DNA	at least 100 ng

The PCR temperature program shown below was used for the promoter amplification:

Table 4: PCR program for the amplification of the *E. huxleyi* promoter region from genomic DNA

Temperature	Time	
94°C	5 min	
94°C	30 s	} 35 cycles
56°C	30 s	
72°C	1 min	
72°C	10 min	
4°C	forever	

2.3.2 Amplification of the resistance gene

The vector pSELECT (InvivoGen, Germany) served as a template for the amplification of the resistance gene *neo* that expresses resistance against the antibiotic G418. A temperature gradient PCR was performed from 51.0 to 57.8°C with the following PCR setup and thermocycler program:

Table 5: PCR setup for the amplification of the resistance gene *neo* from plasmid DNA.

Substance	Amount
Molecular Grade H ₂ O	<i>ad</i> 25.0 µL
5Prime Mastermix	10.0 µL
Primer G418f	1.0 µL
Primer G418r	1.0 µL
Sample DNA	200 ng

The following table shows the PCR temperature program that was used to amplify the resistance gene *neo*.

Table 6: PCR program for the amplification of the resistance gene *neo* from plasmid DNA.

Temperature	Time	
94°C	5 min	
94°C	30 s	} 35 cycles
51°C	30 s	
72°C	1 min	
72°C	10 min	
4°C	forever	

2.3.3 Amplification of the *egfp* gene

The green fluorescence protein gene (*gfp*) is broadly used as a marker for the verification of transformation reactions. The fluorescence of positively transformed clones can easily be observed under the fluorescence microscope. A modification of this gene – enhanced fluorescence green protein (*egfp*) – has already been employed in the available pPha-T1 vector (provided by J. Strauss) for the transformation of *P. tricornutum* from which it could easily be amplified.

Table 7: PCR setup for the amplification of the *egfp* gene from plasmid DNA.

Substance	Amount
Molecular Grade H ₂ O	<i>ad</i> 25.0 µL
5Prime Mastermix	10.0 µL
Primer GenSalf	1.0 µL
Primer GenPstr	1.0 µL
Sample DNA	1.0 µL (unknown conc.)

The temperature program for the amplification of the *egfp* gene is shown in the following table.

Table 8: PCR program for the amplification of the *egfp* gene from plasmid DNA.

Temperature	Time	
94°C	5 min	
94°C	30 s	} 35 cycles
51°C	30 s	
72°C	1 min	
72°C	10 min	
4°C	forever	

2.3.4 PCR product analysis

For the analysis of PCR products an agarose gel electrophoresis was run as described below using the material and equipment stated in the appendix. Concentration measurements were performed with a NanoDrop – Spectrophotometer (Peqlab Biotechnologie GmbH, Germany) using 1-2 μL of the sample.

2.3.5 Gel electrophoresis

PCR products were analyzed in a 1% agarose gel with the addition of 0.01% ethidium bromide in the gel. For gel extraction purposes, DNA was visualized by the addition of the dye SYBR Green to the samples in a ratio of 1:5. The gel extraction was performed with the MinElute Gel Extraction Kit (Qiagen, Germany) according to the instructions in the manual.

2.3.6 Processing of PCR products

For further processing of the PCR products it is useful to clone them into a TOPO vector. It is then easy to amplify sufficient, clean product by growing bacteria. The desired product can be cut out using the induced restriction sites, which are verified at the same time.

The 5Prime Hotmastermix used in the PCR reactions for amplification contains a *Taq* DNA polymerase that adds an additional A to the end of the product resulting in an A-overhang. This overhang can be used to clone the PCR product into a pCR4-TOPO vector which is already provided linearized having a T-overhang

(TOPO TA Cloning Kit for Sequencing, pCR4-TOPO vector, InvitroGen, Germany). For this procedure, fresh PCR product is recommendable, since the additionally added A at the ends of a PCR product is quite fragile and likely to break off after some time. The ligation protocol for electrocompetent cells provided in the manual was followed and a Fast-plasmid-screening (see next section) was performed to check whether the picked clones contain an insert. Sequencing PCRs were conducted as described in 2.4.3.1 to verify the sequence of the single cloned DNA fragments.

2.3.6.1 Fast screening for plasmids with insert

If a great number of clones were picked, a fast screening of the clones for plasmids containing an insert was performed so not each clone had to be prepped. This was done by mixing 5 μ L of Suspension buffer with 5 μ L of Lysis buffer. 10 μ L of the over-night culture was added to the mix and incubated at 99°C for 5 minutes. 3 μ L loading dye were added to the mix which was applied onto a 1% agarose gel containing 0.01% ethidium bromide. As a positive control, the original plain vector was applied to the gel as well, thus a size difference between vectors containing an insert and vectors that do not is notable.

For buffer contents and equipment that was used see appendix.

2.4 Cloning into pUC18

Six restriction sites in the pUC18 MCS were chosen to ligate the promoter region, the resistance gene and the marker gene into the vector (see Fig. 3). For the putative promoter *EcoRI* and *SacI*, for the *neo* gene *BamHI* and *XbaI*, and for the marker *egfp* *SalI* and *PstI* were selected. A schematic drawing of the assembly of the single components is illustrated below.

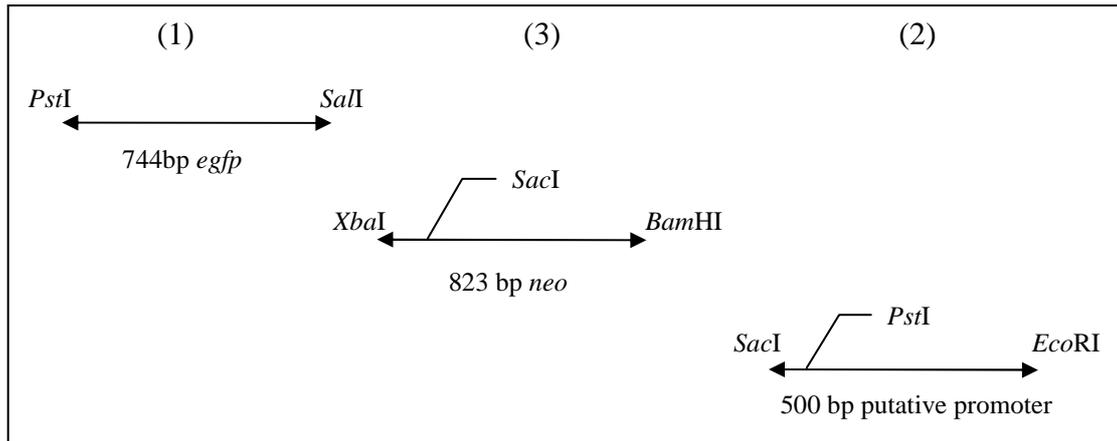


Figure 4: Assembly of single cloning fragments.

Restriction sites used for incorporation of the fragments into the vector and disturbing sites within the fragments are shown. Numbers indicate the predefined order of cloning.

It was realized that the resistance gene also contained a restriction site for *Sac*I and the promoter one for *Pst*I. With this knowledge, some restrictions arose. The cloning order would have to be first cloning the marker *egfp*, then the putative promoter and at last the resistance gene. However, after theoretical alignment of the sequences and looking at the reading frame it was realized that the open reading frame was interrupted and translation into the wanted amino acid sequences could not be sustained. Two transformation vectors were to be created, one containing the putative promoter and the resistance gene, the other with the promoter region and the marker gene. Two transformation experiments were planned. In one, the vector with the resistance selection should be used and in another experiment both vectors should be mixed and transformed at once (personal communication with A. Gruber, University of Constance).

2.4.1 Restriction digestion

For the restriction digestion, a 25 μ L reaction setup was performed as described in the following. Material and equipment was used as stated in the appendix.

Table 9: Reaction setup for restriction digestions.

Substance	Amount
Buffer	2.5 μ L
Restriction enzyme	0.5 μ L each
DNA	approx. 500 ng
BSA (as required)	0.25 μ L
Molecular Grade H ₂ O	<i>ad</i> 25 μ L

The reaction was incubated at 37°C for at least one hour in the heating block or thermocycler.

The restriction digests were purified in a 1% agarose gel stained with SYBR green (see 2.3.5) and extracted from the gel using the MinElute Gel Extraction Kit (Qiagen, Germany). The amount of the extracted DNA fragments was determined running 2 μ L of the extract and 1 μ L loading dye on another 1% agarose gel assessing the amount of DNA by comparison of the sample bands with the DNA size marker bands peqGOLD DNA-Leiter Mix (peqlab, Germany) of known quantity.

2.4.2 Ligation reaction and dephosphorylation

The ligation was carried out using T4 DNA Ligase, provided with 5xDNA Ligase Reaction Buffer (Invitrogen life technologies, Germany). The reaction was run at 15°C over night using the following set up:

Table 10: Reaction setup for ligation.

Substance	Amount
Ligation buffer	4.0 μ L
T4 DNA ligase	1.0 μ L
Insert:Vector	3:1 (at least 3 fmol:1 fmol)
Molecular Grade H ₂ O	<i>ad</i> 20 μ L

After the ligation reaction, 0.5 μ L Calf Intestinal Alkaline Phosphatase (CIAP), provided with 10x Dephosphorylation Buffer and Dilution Buffer (Invitrogen life

technologies, Germany) was optionally added to the digested pUC18 vector reaction in order to prevent religation of the vector. Since the vector was cut with two different restriction enzymes, it should not religate.

The transformation was performed as described in 2.2.5.2, varying the amount of ligation reaction volume added to the electrocompetent TOP10 cells from 0.5 μ L up to 2 μ L.

2.4.3 PCR for sequence verification

PCRs for sequencing verification were performed during several steps of cloning to verify the achieved sequences. After the transformation of *E. huxleyi*, recovery experiments of plasmid sequences were also conducted.

2.4.3.1 Sequencing PCRs

M13 primers (stated in Tab. 11 below) which are general primers, encompassing the insert of the TOPO as well as of the pUC18 vector, were used for sequencing PCRs. The primers were employed at 1 μ M for sequencing PCRs.

Table 11: Sequences of M13 primers used for sequencing.

Name	Sequence	Length [bp]
M13f	5' [AminoC6]GTAAAACGACGGCCAG 3'	16
M13r	5' CAGGAAACAGCTATGAC 3'	17

The sequencing PCR reactions were performed using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) and were purified using the DyeEx 2.0 Spin Kit (Qiagen, Germany) prior to further analysis.

The following reaction setup was used:

Table 12: Reaction setup of a sequencing PCR using M13 primers.

Substance	Amount
Nucleotide Premix	1.0 μ L
5x Sequencing Buffer	1.5 μ L
M13 Primer forward <u>or</u> reverse	1.0 μ L
Sample DNA	100 ng
Molecular Grade H ₂ O	<i>ad</i> 10.0 μ L

The temperature program in Tab. 13 was used for sequencing PCRs.

Table 13: PCR temperature program used for sequencing PCRs.

Temperature	Time	
96°C	1 min	
96°C	10 s	} 30 cycles
52°C	5 s	
60°C	3 min	
4°C	forever	

2.4.3.2 Sequence recovery experiments

M13 and G418 forward and reverse primers were used after biolistic transformation of *E. huxleyi* to see whether the culture contained the constructed vector PnpUC clone 7. For this purpose a different polymerase (Finnzyme, Phusion High Fidelity DNA Polymerase, New England Biolabs, USA) was used since it performs with higher specificity for high complexity templates. 5% DMSO and 1 M betaine was also added to the reactions to improve the performance. All primers were used at a concentration of 10 μ M with the following PCR setup and temperature programs:

Table 14: PCR setup for PnpUC (7) sequence recovery.

Substance	Amount
Molecular Grade H ₂ O	<i>ad</i> 25.0 µL
5x Phusion HF Buffer	5.0 µL
10 mM dNTPs	0.5 µL
Forward primer	1.0 µL
Reverse primer	1.0 µL
DMSO for 5 %	1.25 µL
5 M Betain	5.0 µL
Phusion DNA Polymerase	1.0 µL
Sample DNA	1.0 µL

Table 15: PCR temperature programs for PnpUC (7) sequence recovery with M13¹ and G418² primer pairs using High Fidelity Phusion DNA Polymerase.

Temperature	Time	
98°C	30 s	
98°C	10 s	} 35 cycles
65°C ¹ / 51°C ²	30 s	
72°C	30 s	
72°C	10 min	
4°C	forever	

2.5 Transformation of the microalga

The transformation of the microalga *E. huxleyi* was the final step in this work for the verification of the functioning of the constructed vector PnpUC(7).

2.5.1 Preparation of the cells

An *E. huxleyi* culture was grown in ANT-F/2 containing kanamycin to eliminate bacterial contamination under conditions stated in 2.1. After microscopical inspection of the culture, it was transferred to plain ANT-F/2 medium. The cells were counted and harvested in the exponential phase by centrifuging them in 50 mL tubes at 3000 g for 1 min. The cells were resuspended in ANT-F/2 so that

100 μL contained approx. 3.9×10^7 cells which were spread with a Drigalski-spatula onto the centre of ANT-F/50 Agar plates on the day before bombardment.

2.5.2 Preparation of the DNA

The created transformation vector PnpUC clone 7 was prepared in large quantities using QIAprep Spin Miniprep Kit (Qiagen, Germany) and increasing concentrations using Microcon Centrifugal Filter Devices, Ultracell YM-30 (Millipore, Germany). Approximately 5 μg DNA in 5 μL were added to 50 μL goldparticle (processed according to Kroth, 2007), 50 μL 2.5 M CaCl_2 and 20 μL 0.1 M spermidine (Sigma, Germany). This mix was vortexed for 1 min. at RT, shortly centrifuged, the supernatant was discarded and the pellet resuspended in 250 μL ethanol (100%). The suspension was vortexed again, centrifuged and the pellet was resuspended in a final volume of 50 μL ethanol of which 10 μL was needed for each shot with the particle gun.

2.5.3 Biolistic bombardment

Biolistic particle delivery is a method of transformation that uses helium pressure to introduce DNA-coated microcarriers into cells. Particle delivery is a convenient method for transforming intact cells in culture since minimal pre- or post-bombardment manipulation is necessary (BioRad manual, 1996). The equipment of the biolistic device was prepared and the bombardment performed according to the manual instructions.

2.6 Posttransformational treatment

After bombardment onto the ANT-F/50 agar plates, the cells remained on the unselective plates at standard culture conditions (see 2.1) for them to allow expression of the desired feature (resistance against G418) over night. Cells of all five plates were scraped off with a Drigalski-spatula into 100 mL ANT-F/2 liquid medium provided with 500 $\mu\text{g}/\text{mL}$ G418. This culture was allowed to grow for roughly two weeks until it was investigated microscopically. Half of the culture was plated onto ANT-F/50 plates with and without G418, the other half

transferred into fresh ANT-F/2 liquid medium containing 500 $\mu\text{g}/\text{mL}$ kanamycin and 500 $\mu\text{g}/\text{mL}$ G418.

3. Results

3.1 Growth experiments

Culturing experiments were conducted to understand the growth behaviour of *E. huxleyi* in unselective and selective liquid media, as well as unselective solid media. This is important for future transformation experiments with the microalga.

3.1.1 Growth in liquid media

In selective liquid media conditions according to Strauss (2008), the antibiotics puromycin, chloramphenicol and G418 were utilized. As can be seen in Fig. 5 growth of cultures was inhibited immediately in the presence of G418, chloramphenicol, or puromycin, as opposed to control cultures containing no antibiotics or only kanamycin. Unstressed cultures skip lag phase and directly grow exponentially. They behave very similar, which was also shown in a second growth experiment (data not shown).

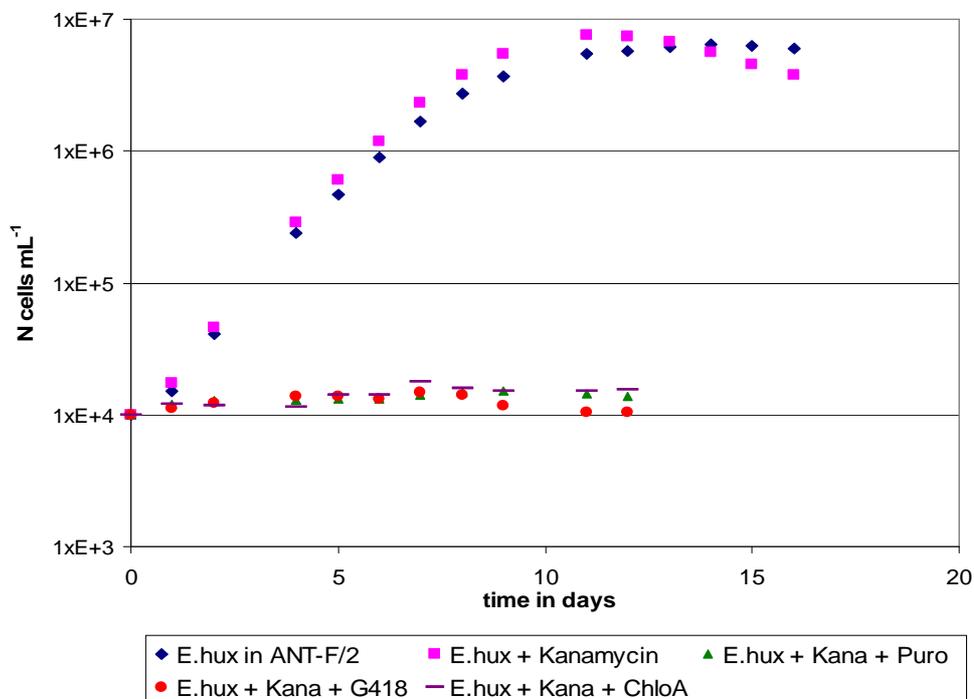


Figure 5: Growth curve of *E. huxleyi* in selective and unselective liquid media with an initial cell count of 10^4 cells/mL.

Displayed are mean cell counts (n=3). Puro = puromycin, Kana = kanamycin, ChloA = chloramphenicol.

The exponential phase with a growth rate of $\mu = 0.84$ for the plain *E. huxleyi* culture in ANT-F/2 and $\mu = 0.77$ for *E. huxleyi* with kanamycin lasted about 9-10 days. A maximum cell count was reached with the culture containing kanamycin at day 11 having 7.4×10^6 cells/mL. Growth of cultures treated with the antibiotics puromycin, chloramphenicol, or G418 was inhibited. The measured number of cells in these cultures, however, remained static and was more or less equivalent to the initial cell count. For stressed cultures the cell count measurement was stopped after 12 days and for unstressed cultures after 16 days since a plateau was reached and cultures containing only kanamycin already reduced in number and seemed to die off.

3.1.2 Growth on solid media

According to Laguna *et al.* (2001) *E. huxleyi* can be grown on solid F/50 medium. Using 1.5 % Bacto Agar in ANT-F/50 medium, very small single colonies could be observed after about 3 days. Inoculation of the plates with a dilution series of cells revealed that plating 1000 cells onto a single agar plate resulted in separated single colonies that can be picked to isolate them.

Regrowth experiments in liquid media were performed by scraping cells off the surface of the plates and transferring them into liquid ANT-F/2. Inspections under the microscope showed that the cells regrown from solid media could be identified as *E. huxleyi* cells (see Fig. 6).



Figure 6: Seven single *E. huxleyi* cells under light microscope with a magnification of 4000.

However, neither regrowth of single picked colonies in liquid ANT-F/2 nor reproduction of the colony growth on solid media could be established.

3.2 Sequence search

The preexisting vector pPha-T1 that is used to transform the diatom *Phaeodactylum tricornutum* initially was chosen as a starting construct. This vector was to be modified to provide it with the resistance gene *neo* against the antibiotic G418 to which *E. huxleyi* is sensitive. The pPha-T1 vector (Fig. 7) contains two promoters and terminators, a zeocin resistance gene (*shble*), pUC ori., and an ampicillin resistance gene (*bla*). The plan was, to replace the second promoter by an *E. huxleyi* promoter and the zeocin resistance by one of the three discovered resistance genes that are suitable for the antibiotics treatment of *E. huxleyi*.

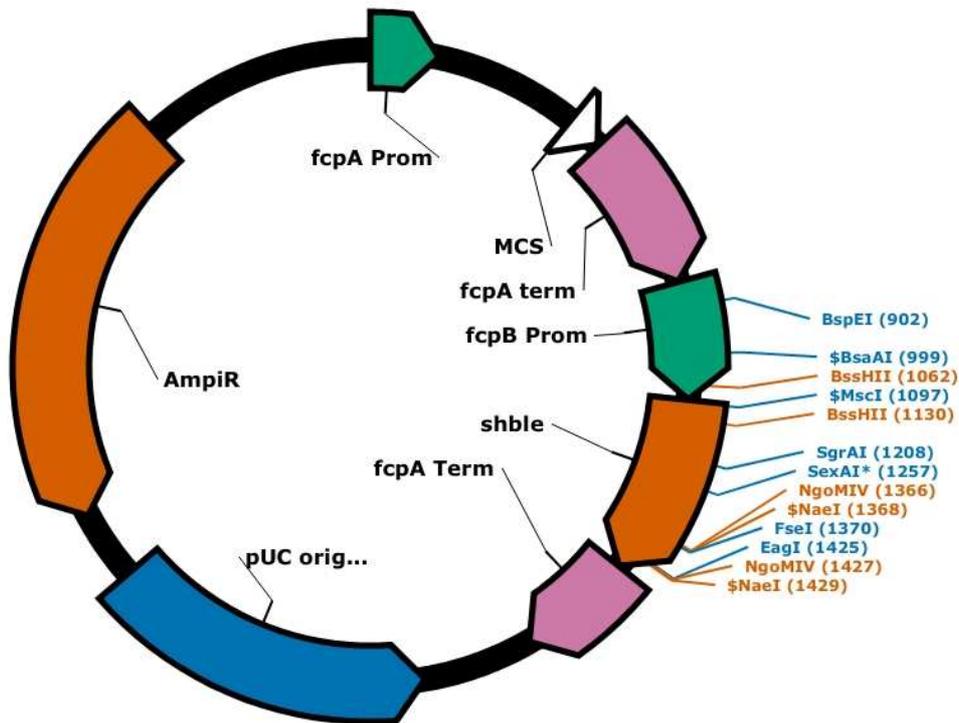


Figure 7: pPha-T1 vector (4095 bp) for the transformation of *P. tricornutum*, showing remaining restriction sites in desired positions.

Resistance genes against ampicillin (Amp^r) and zeocin (*shble*) are indicated as well as pUC ori, the MCS, and terminator and promoter for *fcpA* and *fcpB* genes. Enzymes shown in red cut the vector twice, a \$ indicates enzymes that generate blunt ends and sites depicted with an asterisk will not be cut if the DNA is dam/dcm methylated because of the sensitivity of the enzyme (ANGERSLOUSTAU, 2007).

As can be perceived from Fig. 7 only a few restriction sites could be used for the genetic modification. Most enzymes in the desired position in pPha-T1 cut the vector twice (enzymes depicted in red), occur in the resistance gene, or generate blunt ends and hence are useless for the cloning strategy. Unfortunately, most of the remaining enzymes create identical overhangs, which is why directional cloning is either not possible or restriction enzymes having indefinite restriction sites (containing a Y or W in their recognition site) would have to be used.

The strategy of using a basic pUC18 vector was chosen since this leads to less patchwork on an already modified, restricted vector which means a more straightforward way of work. Basic vectors are also much smaller in size, which facilitates transformation and leaves free capacity for further cloning experiments.

A persistent and highly expressed promoter for *E. huxleyi* is needed for the expression of the resistance gene *neo*. A putative promoter region in front of an *fcp* gene with the protein ID 460117 (EVC02389) (Kegel *et al.*, in press) was selected (for sequence see appendix). This gene is stimulated by light and thereby

regulated, and hence the promoter could later on be used to adjust expression of a desired feature.

3.3 Amplification and optimization of PCR reactions

3.3.1 Optimization of promoter amplification

The PCR conditions for the amplification of the *fcp* promoter had to be optimized to sufficiently amplify the 500 bp putative promoter region. The following figure shows the results of a temperature gradient PCR that was run from 55.0 to 63.1°C with the addition of different DMSO concentrations.

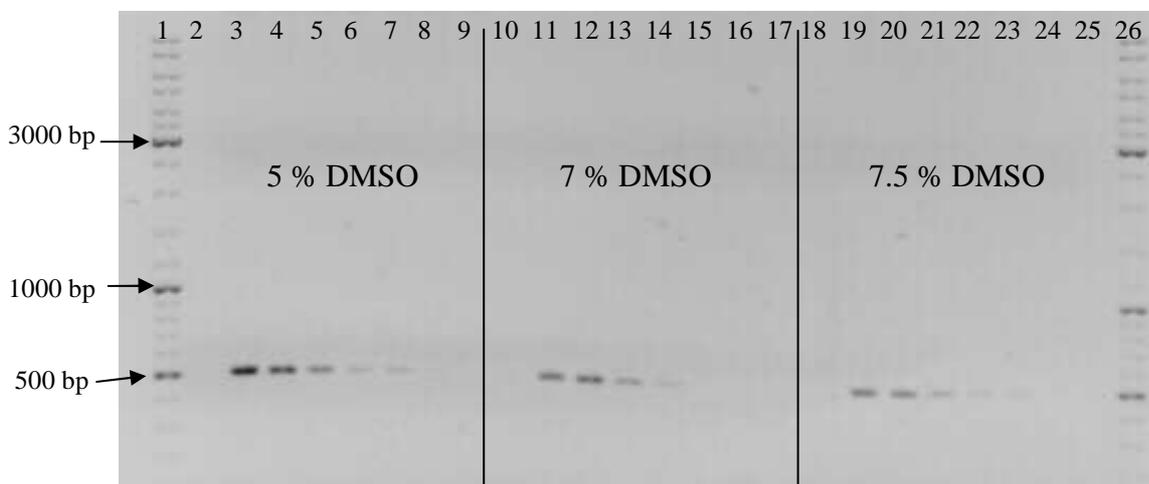


Figure 8: Gel scan of a temperature gradient PCR for the amplification of the *fcp* promoter region. The reaction contains different mastermix setups including the primers FPrf and FPrr for the amplification of the promoter region from genomic DNA. The same sample was amplified at different annealing temperatures (see below) and different DMSO concentrations.

Lane 1+26: Ladder; Lane 2+10+18: neg. control; Lane 3+11+19: 55.2°C; Lane 4+12+20: 55.7°C; Lane 5+13+21: 56.6°C; Lane 6+14+22: 57.8°C; Lane 7+15+23: 59.1°C; Lane 8+16+24: 60.5°C; Lane 9+17+25: 61.8°C.

As can be seen in Fig. 8 amplification using 5 % DMSO at a temperature of 55.2°C yielded the highest amount of PCR product. For further experiments an annealing temperature of 56°C was used and 1 M of the strand separating agent betaine was also added to the reaction mix.

3.3.2 Resistance gene – *neo* – amplification

The amplification of the resistance gene *neo* was performed with primers that already contained the restriction sites for further processing of the PCR products.

A temperature gradient PCR revealed an optimal annealing temperature of approximately 51°C. Since a purified plasmid served as DNA template, no further optimization of the PCR reaction was necessary.

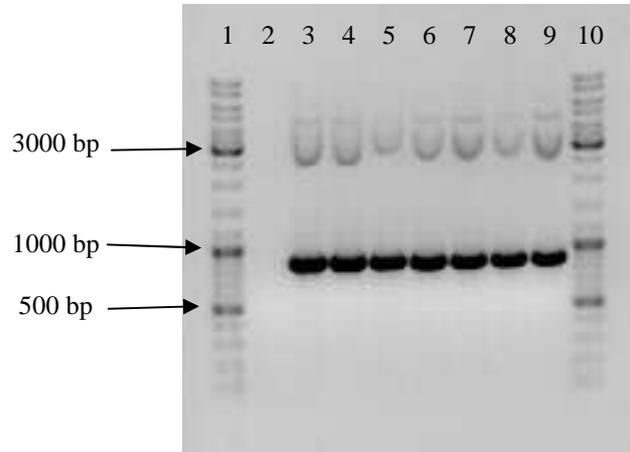


Figure 9: Temperature gradient PCR for the amplification of the resistance gene *neo* from 200 ng of the commercially available plasmid pSELECT (InvivoGen, Germany). Lane 1+10: Ladder; Lane 2: neg. control; Lane 3: 51.2°C; Lane 4: 51.7°C; Lane 5: 52.6°C; Lane 6: 53.7°C; Lane 7: 55.0°C; Lane 8: 56.4°C; Lane 9: 57.8°C.

Fig. 9 reveals that even at an annealing temperature range of roughly 51°C to 58°C, a high yield of PCR product is produced in each reaction. Since products in lane 3 and 4 seemed to display a slightly higher quantity, an annealing temperature of 51°C was chosen for further amplifications.

3.3.3 Marker gene – *egfp* – amplification

For the amplification of the *egfp* sequence, several primer pairs were investigated. After gel purification the primer set, GenSalf and GenPstr, resulted in high amounts of product. The determination of concentrations via gel electrophoresis by comparison to known concentrations of the size marker is depicted in Fig. 10.

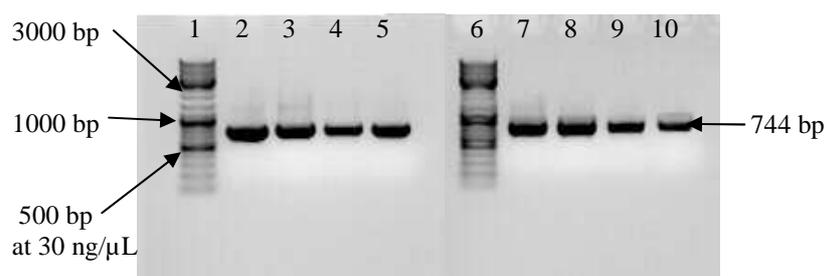


Figure 10: Concentration gel of purified *egfp* product, showing roughly 200 ng/μL PCR product.
Lane 1+6: Ladder; Lane 2-5: sample 1-4; Lane 7-10: sample 5-8.

For this reaction purified plasmid (pPha-T1) served as template DNA, hence, there was no need for further optimization of the reaction. The 744 bp generated PCR product can be identified in the gel scan in Fig. 10.

3.3.4 Fast screening

After cloning into the TOPO vector and transformation of *E. coli* TOP10 electrocompetent cells, a fast screening for plasmids with insert was performed. The same procedure was followed after the performance of cloning experiments using the pUC18 plasmid. A representative gel scan can be seen in the following figure.

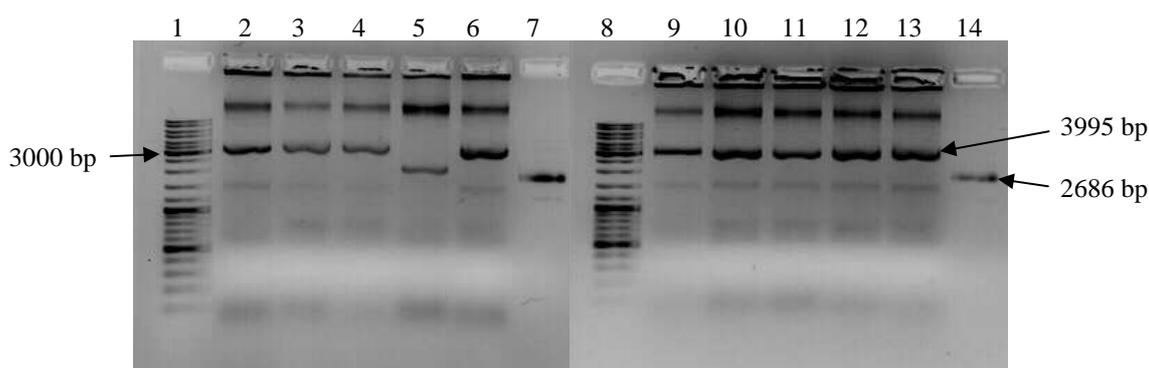


Figure 11: Fast screening of PnpUC clones for plasmids with insert.
Lanes 1+8: Ladder; Lanes 3-6: clones 1-5; Lanes 7+14: pUC 18; Lanes 9-13: clones 6-10.

In Fig. 11 a size difference between the plain pUC18 vector (2686 bp) in lane 7 and 14 and the PnpUC clones (3995 bp) can be clearly distinguished. In this case a plasmid prep of clone 4 (lane 5), containing no insert, did not have to be performed.

3.4 Cloning into pUC18

Cloning of the single components into pUC18 had to be performed in a predefined order (see 2.4). The creation of a newly modified pUC18 vector containing a putative promoter region as well as the resistance gene *neo* against the antibiotic G418 could be proven successful (named: PnpUC). Due to further complications and limited time as well as scope of this work the completion of a vector containing the marker gene *egfp* has to be postponed. The following describes single steps towards vector completion and their validation.

3.4.1 Restriction digestion analysis

After ligating the desired fragments into pUC18 a restriction digestion analysis was performed. This was done by cutting at the respective restriction sites used for incorporation of the fragment.

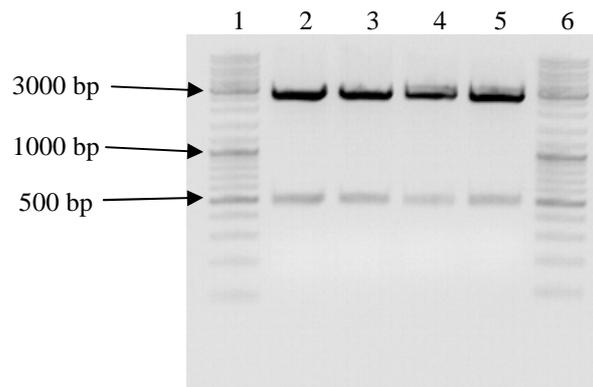


Figure 12: PpUC vector after treatment with *EcoRI* and *SacI* showing the desired promoter as insert (494 bp).

Lanes 1+6: Ladder; Lanes 2-5: clone 1-4

The restriction digestion of *EcoRI* and *SacI* that is depicted in Fig. 12 was performed to show that the putative promoter region comprising roughly 500 bp was successfully incorporated into the pUC18 vector. The remaining pUC18 sequence, 2686 bp in size, can also be seen on the gel scan. This analysis was done with each of the single components to be cloned into pUC18 (data not shown).

3.4.2 Sequencing results

M13 primers comprise the MCS of the pUC18 vector. An insert into the MCS can therefore be sequenced using M13f and M13r primers (see Tab. 11). A sequence alignment of all picked PnpUC clones can be found in the appendix. The alignment revealed, that clone 7 shows 100 % sequence match except for one bp mismatch (substitution of C for a T) in the promoter region at position 1277 that, however, appeared in all nine clones. Clone 7 was therefore the choice for the transformation of *E. huxleyi* via the biolistic bombardment procedure.

3.5 Transformation of *E. huxleyi*

The bombardment of the coccolithophore, intending to transform *E. huxleyi*, was performed once, following the established protocol for the transformation of the diatom *P. tricornutum* (Kroth, 2007). The resulting culture, placed into a 16:8 light:dark cycling cultivation room at 15°C, grew to a milky white broth. 12 days after the bombardment the transformed culture was inspected microscopically.

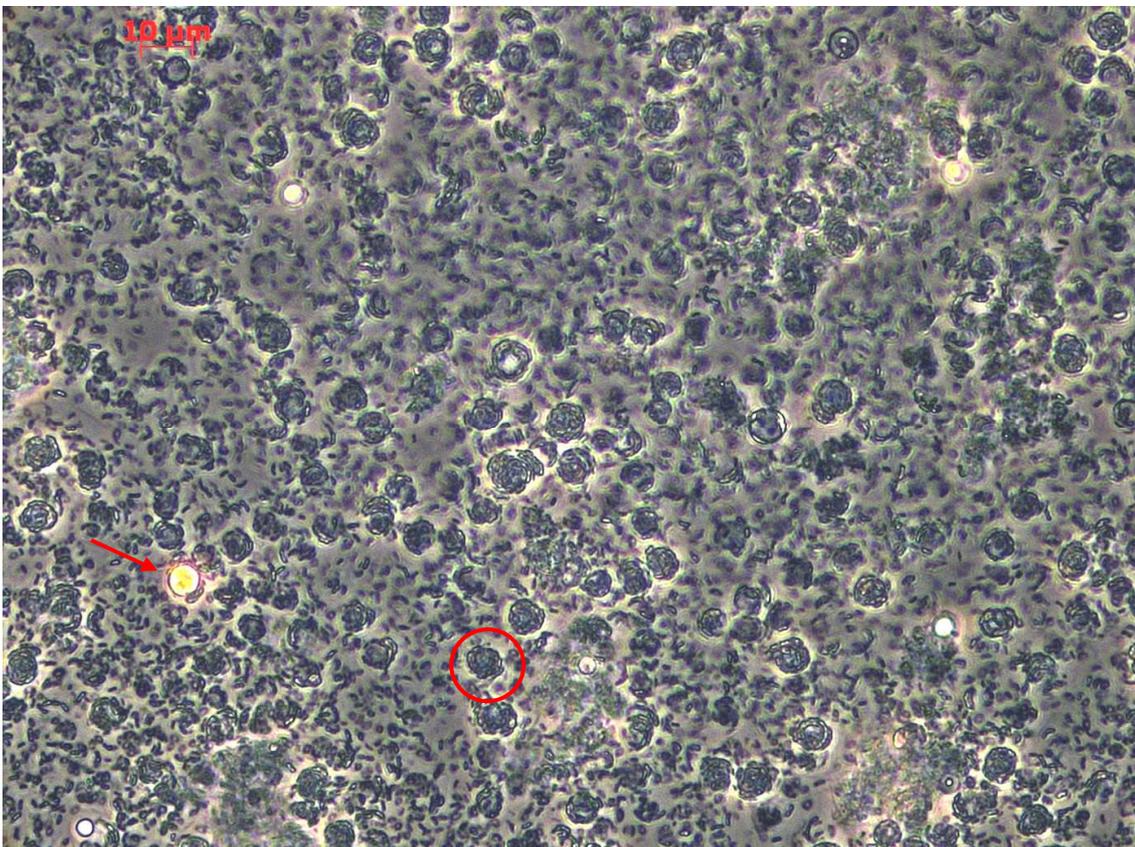


Figure 13: Light microscopical illustration of *E. huxleyi* culture approx. 2 weeks after biolistic bombardment with PnpUC (7) in ANT-F/2 + G418 with a magnification of 4000.

Four naked cells and one having an incomplete coccosphere (see red arrow) could be seen. There were also many detached coccoliths (small particles $\sim 1\text{-}2\ \mu\text{m}$ in size) and empty coccospheres (one depicted with a red circle) in the culture. Since bacteria also seemed to be present (small spots $\sim 1\text{-}2\ \mu\text{m}$ in size, easily confused with coccoliths but moving), the culture was transferred into new liquid ANT-F/2 medium containing G418 and kanamycin.

A growth on solid media could neither be established for non-transformed nor for transformed cultures.

3.5.1 Posttransformational findings

PCR reactions using Phusion High-Fidelity DNA Polymerase and resistance gene primers (G418f and G418r) were performed in order to recapture PnpUC(7) sequences from *E. huxleyi* cultures after bombardment. A concentration dilution series of samples and controls in a PCR (see 2.4.3.2) was performed. Results are illustrated in Fig. 14.

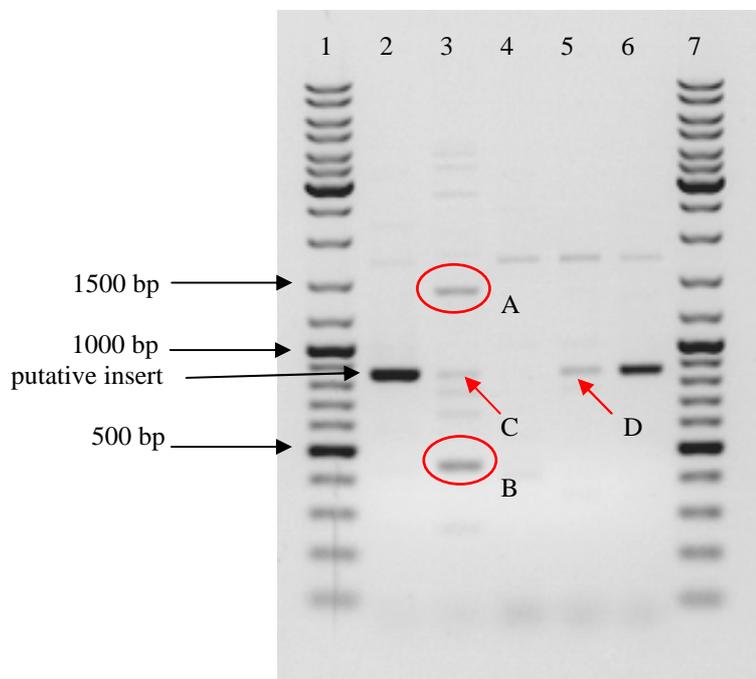


Figure 14: Sequence recovery PCR using resistance gene primers G418f and G418r.

Letter A and B depict fragments that were only observed in the treated culture. Letter C and D mark bands that resemble the size of the putative insert.

Lane 1+7: Ladder; Lane 2: PnpUC(7) at 120 fg + WT DNA at 20 ng; Lane 3: modified *E. huxleyi* DNA at 20 ng; Lane 4: WT DNA at 20 ng; Lane 5: WT DNA at 4 ng; Lane 6: PnpUC(7) at 24 fg + WT DNA at 4 ng.

The positive controls in lane 2 and 6 were spiked with genomic WT DNA in a ratio that demonstrates one gene copy per genome for the plasmid to see whether this amount of genomic DNA would inhibit an amplification. The desired product having 823 bp in length can be detected in lanes 2 and 6 that show positive controls. This PCR reaction reveals several bands in lane 3 (modified genomic *E. huxleyi* DNA) that cannot be seen in the negative control lanes 4 and 5 (WT DNA). One band with the size of the desired insert (about 823 bp) can be observed among these bands in lane 3 (red arrow C). It can, however, also be seen in lane 5 representing WT genomic *E. huxleyi* DNA (red arrow D). Strong DNA fractions that only appeared in the modified *E. huxleyi* DNA are circled in red (A having approx. 1450 bp and B having approx. 450 bp).

After further microscopical inspection of the culture, it was observed that the amount of contaminating bacteria had decreased a lot, however, also only very few *E. huxleyi* cells remained in the culture (estimated amount 100-300 cells/mL). These cells were mostly naked or contained an incomplete coccosphere. Shed coccoliths as well as empty coccospheres had reduced in number as well.

4. Discussion

The introduction of genes into a cell by means of genetic transformation can be a powerful tool for manipulating biochemical pathways, either to gain knowledge of cellular biochemistry or to produce a commercially valuable compound (Dunahay *et al.*, 1995). The ability to express foreign genes integrated into the nuclear genome would be useful for generating a broad spectrum of selectable markers and reporter genes (Kindle & Sodeinde, 1994).

In this work, a novel vector system for the transformation of the coccolithophore *Emiliana huxleyi* was created. The correct assembly of the single components in the vector construct could be proven by sequencing. A transformation experiment of the coccolithophore *E. huxleyi* via biolistic bombardment was carried out and the modified culture was investigated. An alteration of extracted DNA from the treated culture compared to wild type genomic *E. huxleyi* DNA could be detected performing a PCR. The analysis of the dissimilarities between untreated and treated culture has been put aback for this is beyond the scope of this work.

An isolation of transformed clones was not possible, since several requirements such as growth on solid media, that are prerequisites for a successful transformation, are pending.

4.1 Growth experiments

Several growth experiments were performed in the course of this work to verify results that had been discovered in the previous work (Strauss, 2008). The formerly studied antibiotics (puromycin, chloramphenicol, G418) were investigated again and proven to be effective against *E. huxleyi*. Promotion of growth in media containing kanamycin (Strauss, 2008) could not be verified. As can be seen in Fig. 5 growth of the cultures spiked with one of the stated antibiotics is inhibited. However, measurement of initial cell counts could be continued, meaning that the originally introduced cells did not die off. This could be ascribed to the mode of action of these antibiotics that all affect protein biosynthesis (Darken, 1964, Bar-Nun *et al.*, 1983, Pestka, 1971).

Instead of only inhibiting 70S ribosomes of prokaryotic cells, the chosen aminoglycoside G418 also affects 80S ribosomes of eukaryotic cells. By preventing elongation of the translation, incorporation of amino acids into protein

is effectively inhibited. However, it was also investigated, that the maximal degree of translation inhibition obtained with G418 in a cell-free translation system is 90% (Bar-Nun *et al.*, 1983).

Since the life span of *E. huxleyi* under stressed conditions is not known, a survival of cells despite cultivation under selective conditions cannot be excluded. Axenic 2N cultures seem to have very low mortality in stationary phase for up to a month. When bacteria are present, cells start to die off sooner. However, even non-axenic cultures that have been in stationary phase for 6-8 weeks can often be revived (personal communication with P. von Dassow, Station Biologique de Roscoff). Therefore, a second transformation control using a marker gene, such as a fluorescence marker, would be very supportive.

Growth on solid media was investigated at first under conditions studied by Strauss (2008) and later on according to Laguna *et al.* (2001). In this work as well as in Strauss (2008) it could be shown, via microscopical inspection, that regrowth of *E. huxleyi* from solid media in liquid ANT-F/2 media is possible. Laguna *et al.* (2001) describe conditions for the induction of phase switching from the diploid coccolithophore cell (C-cell) to the haploid swarm cell (S-cell) life cycle stages of the marine alga by plating *E. huxleyi* on solid media. The scale bearing haploid life stage (S-cells) of the coccolithophore might be an easier target to be transformed via biolistic bombardment than the diploid life stage of *E. huxleyi* because the C-cells are surrounded by a complete coccosphere. This coccolith construct could be more difficult to be penetrated by microparticles without causing severe damage to the organism. Haploid algae are also favoured for transformation, because the repeat content of the ideal genome, to be transformed, should be as low as possible (Hallmann, 2007). Also, integration of foreign DNA into a haploid genome will be more stable because no second chromosome copy without the insertion would be present. A successful growth on solid media could therefore also induce an advantageous cell state of the microalga which possibly will facilitate further processing of the actual transformation.

The resulting findings of this work, concerning growth on solid media, are not satisfactory since isolated clones on solid media could not be picked and regrown successfully, and therefore not undoubtedly be identified. This precondition has to be established for successful transformation experiments. A separation of

modified cells from non-transformed individuals is a prerequisite for the selection of altered clones. Therefore, further investigations according to Laguna *et al.* (2001) have to be accomplished to attain solid experience in conditioning *E. huxleyi* to growth on solid media.

4.2 Selection of single components

The choice of the single constituents of the transformation vector is a crucial part in the preparatory work.

4.2.1 The promoter

The first requirement is a strong promoter to ensure high levels of mRNA (Wang *et al.*, 2009). Microalgae can recognize and utilize foreign promoter sequences, however, the use of homologous promoters may be necessary to drive expression of foreign selectable markers at sufficient levels to overcome selective pressure (Dunahay *et al.*, 1995).

In addition to dominant selectable markers, there are also several established recessive selectable markers for algal systems. Recessive markers require auxotrophic mutants with mutations in the corresponding endogenous gene and the corresponding intact gene for complementation, but they have the great advantage that a complete endogenous gene with its own promoter is usually used. So, in contrast to many dominant marker constructs, expression and function of the selectable marker construct in the respective organism is quite certain in advance (Hallmann, 2007). A common recessive marker is the nitrate reductase gene (*nit*) which has already been used for functional complementation of nitrate reductase defective mutants of *Chlamydomonas reinhardtii* (Kindle *et al.*, 1989) and a number of other algae (Hallmann, 2007). Similar to the nitrate reductase gene, the *C. reinhardtii* argininosuccinate lyase gene *ASL* was shown to complement mutations in argininosuccinate lyase defective *C. reinhardtii* mutants by selection on arginine-free medium (Debuchy *et al.*, 1989).

In another unicellular green alga, *Chlorella vulgaris*, a resistance gene was expressed under the control of the cauliflower mosaic virus promoter (CaMV35S) for selection with hygromycin (Chow & Tung, 1999). The CaMV35S promoter is a typical promoter for strong expression in transgenic higher plants (Hallmann,

2007). Chimeric constructs of endogenous promoters, fusion promoters, or virus promoters with mutant forms have successfully been reported. SV40 is a polyomavirus that is found in both monkeys and humans, however this promoter still works in a brown alga (Hallmann, 2007).

The choice of an endogenous *fcp* (fucoxanthin, chlorophyll a/c-binding protein gene) putative promoter region enhances the likelihood of its viability in this organism and additionally reveals the opportunity for later regulation of the expression of a desired introduced target gene in the vector.

It has to be considered though, that inserted resistance genes regulated by an *fcp* promoter region are expressed most sustainable with optimal light conditions. The ideal conditions for the best capacity of the promoter and hence, the expression of the resistance gene, have to be found out. The efficiency of the promoter strongly varies from the light intensities and the different spectra (personal communication with P. Kroth, University of Constance). It is reported, that protein accumulation from the same promoter may vary as much as 10000 fold depending on the choice of translation control signals (Eibl *et al.*, 1999, Zou *et al.*, 2003, Kuroda & Maliga, 2001).

Diatom *fcp* proteins, for instance, are functionally equivalent to chlorophyll a/b-binding proteins (CAB) of higher plants and green algae. Protein synthesis of CAB has frequently been shown to be controlled by light quality and quantity, and this regulation acts primarily at the level of gene expression (Falciatore *et al.*, 1999, Terzaghi & Cashmore, 1995).

Best *fcp* promoter performance is likely to be achieved under permanent light conditions. *E. huxleyi* cultures transformed via biolistic bombardment were, however, kept under 16:8 light:dark cycling conditions which were standard culturing conditions during this work. As shown in the work of Oeltjen (2004), cultures have to be adapted to permanent light for an extended period of time as done with *Phaeodactylum tricoratum* (Apt *et al.*, 1996) since gene expression is apparently also regulated in a circadian rhythm (Oeltjen *et al.*, 2004).

The amplification of the putative promoter region required the addition of strand separating agents such as DMSO and betaine (Frackman *et al.*, 1998). According to findings of Baskaran *et al.* (1996) this suggests a high GC content in the *E. huxleyi* genome which has likewise been discovered in other microalgae (Jarvis *et al.*, 1992).

Posttranscriptional gene silencing, *e.g.* through methylation caused by positional effects and epigenetic mechanisms, as well as RNA editing, incomplete promoters, enhancers or other regulatory sequences, insufficient DNA delivery, or unsuccessful integration into the genome may also contribute to failure of foreign gene expression (Strauss, 2008, Wang *et al.*, 2009, Hallmann, 2007).

4.2.2 The resistance gene - *neo*

Genes that confer resistance to antibiotics have been used successfully as selectable transformation markers for a wide variety of organisms (Dunahay *et al.*, 1995). They confer a new trait to any transformed target strain of a certain species, no matter of the respective genotype. By far the highest number of selectable marker genes have been established for *C. reinhardtii* (Hallmann, 2007).

The choice of G418 as selective antibiotic are intensively investigated and discussed in Strauss (2008). There are several aspects that should be considered when continuing with *E. huxleyi* transformation and selective growth experiments. As was suggested by Strauss (2008) cells grown in a less saline medium might increase the sensitivity of *E. huxleyi* towards G418 as could be shown with the diatoms *Cyclotella cryptica* and *Navicula saprophila* (Dunahay *et al.*, 1995, Kuroda & Maliga, 2001), as well as *P. tricornutum* (Zaslavskaja *et al.*, 2000). These non ideal conditions normally affect cell growth in a negative way (Brand, 1984). Oceanic *E. huxleyi* strains can, however, grow down to at least 25 psu (Brand, 1984). The *neo* resistance gene, expressing amino 3'-glycosyl phosphotransferase, which confers resistance to G418, is used extensively in gene transfer to mammalian cells (Valera *et al.*, 1994). Nevertheless, Hasnain *et al.* (1985) have employed *neo* in the transformation of *C. reinhardtii*. For the operation as a selective agent in other transformation systems such as the diatoms *P. tricornutum* (Zaslavskaja *et al.*, 2000), *Cyclotella cryptica* and *Navicula saprophila* (Dunahay *et al.*, 1995), but also the microalga *C. reinhardtii* (Bingham *et al.*, 1989), the resistance gene *nptII*, expressing neomycin phosphotransferase II which also confers resistance to G418, has been proven successful. Valera *et al.* (1994) suggest that *neo* gene expression may induce alterations in gene expression and metabolism of modified cells. This and the high GC content mentioned above suggest an examination of the *E. huxleyi* genome to investigate a preferred codon usage. Such an investigation should be considered

for future transformation studies since a species specific codon bias may hinder expression of foreign genes (Jarvis *et al.*, 1992, Kindle & Sodeinde, 1994, Dunahay *et al.*, 1995). This effect could be studied by the construction of a synthetic gene with appropriate codon usage.

The alterations in gene expression and metabolism, the species specific codon usage, and the fact that the *neo* gene is known to be favoured for the transformation of mammalian cells, implies, that the *neo* gene might preferentially be substituted by the *nptII* gene that has already been proven effective for other algae transformation systems (Bingham *et al.*, 1989, Dunahay *et al.*, 1995, Zaslavskaja *et al.*, 2000).

4.2.3 The marker gene - *egfp*

For some applied research like reconstruction or analysis of biochemical pathways or regulatory systems, identification of transcription regulatory sequences, locating subcellular specific proteins, the availability of several selection markers is useful. Reporter genes often code for enzymes that convert a substrate into a coloured product, or result in light emission, or the reporter gene product is a fluorescent protein itself (Hallmann, 2007). Commonly used reporter genes include *uidA* (β -glucuronidase) and *gfp* (green fluorescent protein). The *gfp* gene is expressed endogenously by the jellyfish *Aequorea victoria* (Prasher *et al.*, 1992). Several modifications have been made to this gene to enhance its expression in specific organisms as well as the fluorescent characteristics of the expressed protein. The *gfp* gene is widely used for cell biology studies since the expression and targeting of the protein product within the cell can be directly visualized. The *egfp* (enhanced green fluorescent protein) gene has 190 silent mutations to ensure optimum codon usage and expression in human cells (Zaslavskaja *et al.*, 2000). Coincidentally, the *egfp* gene has been engineered to employ codons similar to those preferred by the diatom *P. tricornutum* (Bhaya & Grossman, 1993). The effects of codon bias are very well known in other systems and can affect protein production by several orders of magnitude as discussed above (Zaslavskaja *et al.*, 2000). A known preferred codon usage for *E. huxleyi* would therefore be supportive if the use of marker genes such as *gfp* or *egfp* is desired.

A very valuable application of the *gfp* marker is the construction of fusion proteins for the location or identification of proteins within the cell (Apt *et al.*, 2002, Gruber *et al.*, 2007, Kilian & Kroth, 2005). For this application, the construction of a fusion protein resulting from the *neo* and the *egfp* genes required a very precise assembly of the single components into the vector.

4.3 Cloning and transformation strategy

The cloning strategy of creating one vector comprising promoter, resistance and marker gene could not be realized. An interruption of the open reading frame between the resistance gene *neo* and the marker gene *egfp* interfered with the initial cloning strategy. Therefore, two vectors, one containing the *fcf* promoter and the selective resistance gene *neo*, and another holding the putative promoter plus the marker gene *egfp* were to be designed. Co-transformations of two vectors one possessing a selective, the other a marker gene have successfully been accomplished (Kindle, 1990, Poulsen *et al.*, 2006, Dunahay *et al.*, 1995, Falciatore *et al.*, 1999). Using a 1:1 mixture of both plasmids, Poulsen *et al.* (2006) could demonstrate an 80% efficiency of co-transformation.

A number of time consuming drawbacks lead to the construction of only one plasmid, PnpUC, containing the putative *fcf* promoter region and the resistance gene *neo* for the selection with the antibiotic G418. According to sequencing results all essential components of the single fragments such as start (ATG) and stop codon (TAA) are included in the novel construct (see appendix). In all clones one bp mismatch could be observed (T replaced by C), which is positioned in the promoter region and therefore does not induce a change in transcribed protein sequences. Since all sequenced clones show that mismatch, it implies that there is a sequence mistake in literature, a variation in previously performed sequencing reactions.

The basis of almost all algal transformation methods is to cause temporal permeability of the cell membrane, enabling DNA molecules to enter the cell. Entrance of the DNA into the nucleus and integration into the genome occurs without any external help. DNA integration mainly occurs by illegitimate recombination events, resulting in ectopic integration of the introduced DNA and, thus, leading to stable genetic transformation (Hallmann, 2007).

Several methods have been reported for introducing DNA into the nucleus, including particle bombardment (Debuchy *et al.*, 1989, Kindle *et al.*, 1989, Klein *et al.*, 1987), electroporation (Brown *et al.*, 1991), and agitation with glass beads (Kindle, 1990) or silicon fibers (Dunahay, 1993).

Microparticle bombardment seems to work, in principle, with any type of cell regardless of the consistency and rigidity of the cell wall. Hallmann (2007) therefore recommends it as the method to start with if someone intends to produce transgenics with a previously untransformed alga, especially if the functionality of utilized promoters and selectable markers or other experimental issues are uncertain. The penetrating power of the microprojectiles can be increased without difficulty, such that even the tough silica cell walls of diatoms do not form an impenetrable barrier (Hallmann, 2007).

Two important parameters in microprojectile bombardment are the size of the particles and the He pressure used for the acceleration of the particles (Poulsen *et al.*, 2006). Since *E. huxleyi* is the first coccolithophore to be transformed the nature of the coccosphere might require a completely different particle size and acceleration speed as described in the implemented protocol established for the diatom *P. tricornutum* (Kroth, 2007). The most suitable parameters for particles to enter *E. huxleyi* have to be investigated. This could be achieved by bombarding the coccolithophore with particles coated with dye that could be redetected by microscopical inspection of the culture after microprojectile delivery. Similar investigations have already been accomplished at the Marine Laboratory in Plymouth where fluorescent dyes have been introduced into eukaryotic cells via biolistic bombardment (personal communication with M. Cock, Station Biologique de Roscoff). Successful penetration of particles into the cells could be observed for the flowering plant *Commelina communis*, cells of *C. reinhardtii*, and zygotes of the brown alga *Fucus serratus* (Bothwell *et al.*, 2006). In the study of Bothwell *et al.* (2006) gold particles instead of tungsten particles as utilized in most other works (Klein *et al.*, 1987, Diener *et al.*, 1990, Kindle *et al.*, 1989, Debuchy *et al.*, 1989) were used. Gold particles were also used in this presented work since they yielded higher transformation rates in recent experiments (Strauss, 2008). One disadvantage of gold particles is their colour that almost resembles the colour of the *E. huxleyi* culture on the agar plates. Therefore, a visual inspection of the plates after bombardment does not reveal a preliminary

success at first glance. As mentioned above, the He pressure, with which the particles are accelerated, as well as the use of most suitable rupture disks has to be optimized for microprojectile delivery into *E. huxleyi*. In the performed experiment sufficient pressure for the utilized rupture disks could not be established because the disks busted before a certain pressure level was achieved. A positive outcome of this experiment was therefore very questionable. The utilized vector, PnpUC(7), had been newly created for the transformation of *E. huxleyi* with a customised promoter that would only express the selective gene in this organism. A positive control for the transformation thus could not be realised. Among others a verification of the bombardment procedure according to Kroth (2007) was, however, conducted several times by Strauss (2008) and M. Bayer (Alfred-Wegener Institute, Bremerhaven) using the established transformation system pPha-T1 (Zaslavskaja *et al.*, 2000) for the diatom *P. tricornutum*. Further studies of most applicable particles, their properties and acceleration speed suitable for the coccolithophore *E. huxleyi* need to be done.

4.4 Transformation experiment of *E. huxleyi*

After biolistic bombardment the bombarded *E. huxleyi* culture was kept in selective liquid media (ANT-F/2 + G418) under standard culturing conditions (see 2.1) where it grew milky white (see Fig. 13). This clouding of the culture can be caused by a shedding of coccoliths from the coccolithophore which usually appears in stressed cultures (Balch *et al.*, 1992). Balch *et al.* (1992) reported an inverse relation between coccolith and nutrient concentrations. The production and detachment of coccoliths by *E. huxleyi* is also strongly dependent on the growth phase of the cells. Coccoliths begin rapidly detaching just prior to and continuing well into stationary phase (Balch *et al.*, 1992). It can be concluded that growth conditions for the culture were non-ideal at the time after bombardment. The 16:8 light:dark cycle may have prevented the *fcp* promoter from expressing the *neo* gene permanently, so that during eight hours darkness per day the culture was exposed to the antibiotic G418.

Inspection of the modified culture revealed a large contamination by bacteria in spite of the presence of the antibiotic G418. Further transfer of the culture into fresh liquid ANT-F/2 including G418 and kanamycin lead to a dilution of the *E. huxleyi* cell concentration since the cells did not proliferate any longer.

Nevertheless, a reduction of contamination, which could be observed at another microscopical inspection, was achieved after the addition of both antibiotics, G418 and kanamycin. Proliferation of the cells can be disturbed since incorporation of the DNA into the nuclear genome apparently occurs randomly, so that the transforming DNA may integrate into an active gene and thereby disrupt its function (Kindle & Sodeinde, 1994). A sustainable survival of the modified culture could hence not be achieved.

As mentioned before, the rate of transcription can be influenced by transcriptional enhancer or repressor elements located within introns (Goto *et al.*, 1996, Brooks *et al.*, 1994). Epigenetic suppression of gene expression, inefficient transcription due to the lack of appropriate promoter and/or enhancer elements, the lack of introns required for efficient RNA processing (Kindle & Sodeinde, 1994, Stevens *et al.*, 1996), and silencing of introduced genes by methylation (Blankenship & Kindle, 1992) were possible reasons for the failure to express heterologous genes in *Chlamydomonas* (Lumbreras *et al.*, 1998). Elements such as intronic enhancer elements have typically been located within the first intron of eukaryotic genes (Koziel *et al.*, 1996). Taylor (1997) has stated the importance of such intragenic elements in the regulation of plant gene expression. Insertion of *Chlamydomonas* introns within the resistance gene sequence significantly increased the expression of the gene demonstrating that introns play an important role in the efficient expression of eukaryotic genes (Lumbreras *et al.*, 1998). Kindle & Sodeinde (1994) note that in *Chlamydomonas* consistent success in nuclear transformation was not achieved until endogenous genes were used as selectable markers. Hence, it has to be considered that a complete reconstruction of the vector PnpUC(7), used for the transformation of *E. huxleyi* in this work, might be necessary.

4.4.1 PCR results

PCR reactions performed with the extracted DNA of the modified culture revealed dissimilarities when compared to WT genomic DNA. For an easier discussion, the outcomes of the PCR reactions are again displayed in Fig. 15. From PCR results, a difference between the treated and the WT culture is evident, since a number of new PCR products can be detected in the modified culture (*e.g.* letter A with approx. 1450 bp and B with approx. 450 bp in Fig. 15). Fragments, resembling the putative insert in size (*neo* gene having 823 bp amplified by G418f

and G418r) can be observed amongst all samples, except in lane 4 (WT DNA). Another concentration of the WT DNA (lane 5), however, also reveals a fragment having the size of the desired insert, suggesting unspecific amplification of genomic DNA.

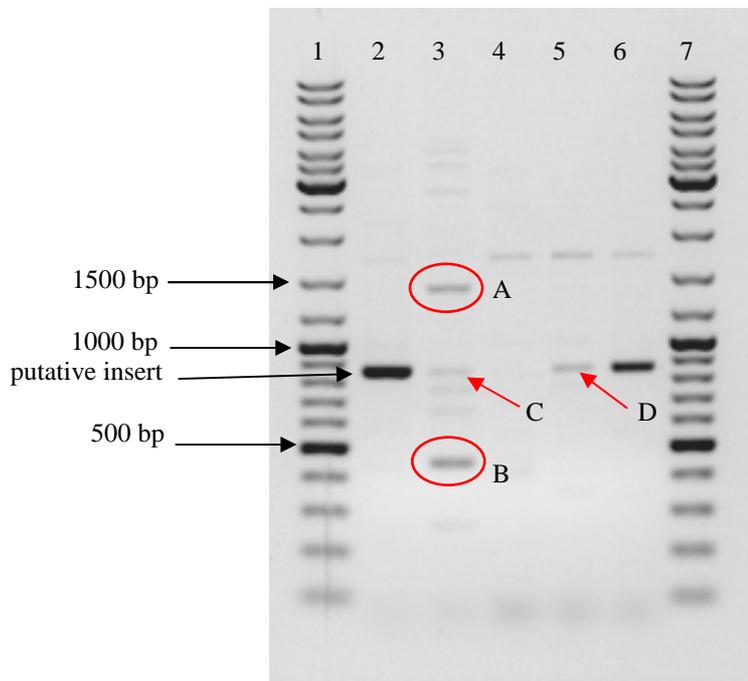


Figure 15: Sequence recovery PCR using resistance gene primers G418f and G418r.

Letter A and B depict fragments that were only observed in the treated culture. Letters C and D mark bands that resemble the size of the putative insert.

Lane 1+7: Ladder; Lane 2: PnpUC(7) at 120fg + WT DNA at 20 ng; Lane 3: transformed *E. huxleyi* DNA at 20 ng; Lane 4: WT DNA at 20 ng; Lane 5: WT DNA at 4 ng; Lane 6: PnpUC(7) at 24 fg + WT DNA at 4 ng.

As integration of the transforming DNA into the genome occurs by coincidence (Kindle & Sodeinde, 1994), any broken fragment of the PnpUC(7) vector might have been incorporated. Fragments containing a reasonable annealing site for the utilized primers should be represented by the unknown bands. Kindle & Sodeinde (1994) suggest that most transformation events occur at nonhomologous genomic locations and that transformants generated by particle bombardment contain multiple copies of the transforming DNA (Debuchy *et al.*, 1989, Kindle *et al.*, 1989, Diener *et al.*, 1990). Unknown, new fragments in the modified culture, that do not resemble the size of the desired insert, could hence also depict multiple copies of transformed DNA sequences.

Restriction digestion analysis, followed by Southern blot of resulting fragments was realised in most studies after transformation of an algae (Dunahay *et al.*,

1995, Zaslavskaja *et al.*, 2000, Hasnain *et al.*, 1985, Sun *et al.*, 2006). Here, PCR reactions were conducted, producing fragments that need to be isolated, purified and sequenced for their identification.

In this work, clues for a possible transformation of *E. huxleyi* are presented. For clarification we should further investigate the nature of the change that took place in the treated culture. Hence, sequencing analysis of the novel fragments in the modified genomic DNA should be readily performed. However, further analysis of the discrepancies between WT and modified genomic DNA unfortunately lies beyond the scope of this work.

4.5 Outlook

In this work, first steps towards the transformation of *E. huxleyi* were realized. To improve transformation, subsequent steps have to be accomplished.

At first, the alterations that occurred in the modified culture have to be analyzed by repeating the amplification of the desired insert from the treated culture as well as from untreated genomic DNA. The generated bands of appropriate size should be purified from the gel and cloned into a vector from which they can be sequenced using general primers. Regardless of the template DNA that was used, sequencing should be performed with all bands of appropriate size that form. The fragments can then be identified and it can be seen whether unspecific amplification occurs.

If the transformation was unsuccessful and no vector sequences can be recaptured from the modified cultures, there are numerous possibilities, that have been discussed here, that could have lead to this failure and can be investigated.

A recapturing of vector sequences from a transformed *E. huxleyi* culture has not been presented before. The transformed culture would have to be kept alive and growing under selective conditions in order to breed transformed clones. A method for the separation of single clones by plating on solid media still has to be established. The expressed antibiotic resistance, amino 3'-glycosyl phosphotransferase, and/or the produced RNA from the *neo* gene, has to be purified and analyzed via Western and/or Southern Blot for verification as done in other transformation experiments (Apt *et al.*, 1996, Zaslavskaja *et al.*, 2000, Hasnain *et al.*, 1985, Dunahay *et al.*, 1995, Falciatore *et al.*, 1999).

The transformation method has to be optimized to attain higher transformation efficiencies. A second vector bearing a marker gene should be implemented. The co-transformation of the two vectors into *E. huxleyi* should facilitate an identification of positively transformed clones. An investigation of desired genes, such as genes playing an important role in viral infection, can then begin.

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Appendix

Material and Equipment

Growth experiments:

Description / Name	Supplier	Cat.No.
Bacto Agar	Becton, Dickinson and Company	214050
Chloramphenicol BioChemical G418	AppliChem	A1806,0025
Kanamycin-sulfat	InvivoGen	ant-gn-5
Inkubator 1000, Unimax 1010	Omnilab	2.700 086
Inkubator 1000, Unimax 1010	Heidolph Instruments	549-90010-00 / 543-12310-00
Multisizer TM 3 Coulter Counter, Coulter Isoton II Diluent, 20 mL Coulter flasks	Beckman Coulter GmbH, Deutschland	
Puromycin	InvivoGen	ant-pr-1

Preparation of Guillard's f-solution for cultivation of *Emiliana huxleyi*:

Composition of stocks:

Nutrient salt stock solutions (all stocks were prepared with Milli-Q-water):

- 1) NaNO₃:
- 2) Na₂HPO₄ * 2H₂O:
- 3) Na₂SiO₃ * 9H₂O:
- 4) Trace metal solution

Stock solution for trace metal solution:

- | | | |
|----|--|----------------|
| a) | ZnSO ₄ * H ₂ O | 0.150 g/100 mL |
| | CuSO ₄ * 5H ₂ O | 0.100 g/100 mL |
| | CoSO ₄ * 7H ₂ O | 0.120 g/100 mL |
| | MnSO ₄ * H ₂ O | 2.000 g/100 mL |
| b) | FeCl ₃ * 6H ₂ O | 5.000 g/100 mL |
| c) | Na ₂ MoO ₄ * 2H ₂ O | 0.065 g/100 mL |
| d) | Na ₂ EDTA (Titrplex III) | 5.000 g/100 mL |

Composition of trace metal solution

- a) 10 mL

b)	10 mL
c)	10 mL
d)	10 mL
Milli-Q-water	<i>ad</i> 1000 mL

Stock solutions **1) – 4)** were autoclaved.

5) Vitamin solution

Stock solutions:

a) Biotin	1 mg/10 mL
b) Vitamin B12	1 mg/10 mL

Stock solutions were sterilized by filtering through a 0.2 µm filter and stored at -20°C.

To prepare the vitamin solution a volume of 1 mL of each stock was added to 100 mL Milli-Q-water. Finally 20 mg thiamine HCl were added. The ready vitamin solution was sterile filtered through a 0.2 µm filter and aliquots of 10 mL were frozen at -20°C.

Composition of ANT-F/2 medium:

Antarctic seawater	1000 mL
Stock solution 1 (NaNO ₃)	1 mL
Stock solution 2 (Na ₂ HPO ₄)	1 mL
Stock solution 3 (Na ₂ SiO ₃)	1 mL
Stock solution 4 (trace metalsolution)	1 mL
Stock solution 5 (vitamin solution)	1 mL

Medium was sterilized by filtration through Sartobran capsula (Sartorius, Germany) using a 0.2 µm final filter.

DNA-Isolation:

Description / Name	Supplier	Cat.No.
Microcentrifuge 5417R	Eppendorf	5407 000.317
Centrifuge 5810R	Eppendorf	5811 000.010
DNeasy Plant Mini Kit	Qiagen	69104

Preparation of backups:

Description / Name	Supplier	Cat.No.
Agar - Plant cell culture tested	Sigma	A 1296-500G
BioPhotometer	Eppendorf	6131 900.102
Centrifuge 5810R	Eppendorf	5811 000.010
Electroporator, Gene Pulser Xcell	BioRad	165-2666
Electroporation Cuvette	Molecular Bio Products	5510
Glycerol for molecular biology	Sigma	G5516-1L
Inkubator 1000, Unimax 1010	Heidolph Instruments	549-90010-00 / 543-12310-00
BioPhotometer	Eppendorf	6131 900.102
Sodium Chloride	Sigma	S3014-1KG
D-Sorbitol	Sigma	S-1876-1KG
Streptomycinsulfat	AppliChem, BioChemica	A1852.0025
TOP10 Electrocompetent <i>E. coli</i> cells	Invitrogen	
Tryptone enzymatic digest from Casein	Sigma	95039 1kg
X-Gal	promega	V3941
Yeast extract	Omnilab Life Science	2.700 165

Preparation of electrocompetent cells:**LB-medium:**

- 10 g Tryptone
- 5 g Yeast extract
- 10 g sodium chloride
- add to 1 L dH₂O

Washing Buffer:

- 1 L dH₂O
- 10 % Glycerol

Suspension Buffer:

- Washing Buffer
 - 2.5 % Sorbitol
- filter sterilize

Fast screening for plasmids with insert:

Description / Name	Supplier	Cat.No.
EDTA	Sigma	E-5134
RNase A	Qiagen	1018048
SDS	Sigma	L-4390
Sodium Chloride	Sigma	S3014-1KG
Tris-HCl	Ambion	AM9855G

Suspension Buffer:

- 50 mM Tris-HCl
- 10 mM EDTA
- pH 8.0 (25 °C)
- RNase A (2.5 mg in 25 mL)

Lysis Buffer:

- 0.2 M NaOH
- 1 % SDS

(equipment see PCR product analysis)

Plasmid preparation:

- QIAprep Spin Miniprep Kit (Qiagen, Germany, Catalogue # 27106)
- Microcentrifuge 5417R (Eppendorf, Germany, # 5407 000.317)

PCR reactions for amplification:

Description / Name	Supplier	Cat.No.
Mastercycler / Mastercycler gradient	Eppendorf	5333 000.018 / 5331 000.010
5 Prime Hotmastermix 2.5x	5 Prime	2200410
Betaine	Sigma	B-2629
Dimethyl sulfoxide, for molecular biology (DMSO)	Sigma	D8418-100ML
Molecular Biology Grade H ₂ O	Eppendorf, Germany	0032.006.159
pSELECT	Invivogen, Germany	Cat.No. psetn-mcs

PCR product analysis:

Description / Name	Supplier	Cat.No.
Agarose – LE	Ambion	AM9040
Bromphenol Blue sodium salt, for electrophoresis Molecular biology tested	Sigma	B5525-10G
DNA-Leiter Mix	peqGOLD	25-2040
Elektrophoresechamber Sub-Cell GT, Mini-Sub-Cell GT	BioRad, Germany	
Ethidium Bromide	Sigma	E-1510
Glycerol for molecular biology	Sigma	G5516-1L
Loading buffer	Applied Biosystems, USA	
NanoDrop - Spectrophotometer	Peqlab Biotechnologie GmbH	ND-1000
Power supply: Power Pac Basic	BioRad, Germany	
Photo-Imager	Vilber Lourmat, France	
Safe Imager	Invitrogen	
Software BioCapt Version 11.02		
SYBR Green I nucleic acid gel stain	Molecular Probes, Eugene	S7563

50x TAE-buffer:

- 2 M Tris-Acetate
- 0.05 M EDTA
- pH 8.0 (25 °C)

Processing of PCR products and restriction digestion:

- TOPO TA Cloning Kit for Sequencing, pCR4-TOPO vector, Invitrogen, Germany, Catalogue # 45-0071
- MinElute Gel Extraction Kit, Qiagen, Germany, Catalogue # 28606

Description / Name	Supplier	Cat.No.
<i>Bam</i> HI, provided with NEBuffer3 and BSA	New England Biolabs Inc.	R0136
Calf Intestinal Alkaline Phosphatase (CIAP), provided with 10x Dephosphorylation Buffer and Dilution Buffer	Invitrogen life technologies	18009-019
<i>Eco</i> RI, provided with NEBuffer <i>Eco</i> RI	New England Biolabs Inc.	R0101
<i>Pst</i> I, provided with NEBuffer3 and BSA	New England Biolabs Inc.	R0140
<i>Sac</i> I, provided with NEBuffer1 and BSA	New England Biolabs Inc.	R0156
T4 DNA Ligase, provided with 5xDNA Ligase Reaction Buffer	Invitrogen life technologies	15224-025
<i>Xba</i> I, provided with NEBuffer4 and BSA	New England Biolabs Inc.	R0145
Mastercycler / Mastercycler gradient	Eppendorf	5333 000.018 / 5331 000.010
Thermomixer comfort	Eppendorf	5355 000.011

Ligation Reaction and dephosphorylation:

- T4 DNA Ligase, provided with 5xDNA Ligase Reaction Buffer, Invitrogen life technologies, Germany, Catalogue # 15224-025

- Calf Intestinal Alkaline Phosphatase (CIAP), provided with 10x Dephosphorylation Buffer and Dilution Buffer (Invitrogen life technologies, Germany, Catalogue # 18009-019)

Description / Name	Supplier	Cat.No.
Mastercycler / Mastercycler gradient	Eppendorf	5333 000.018 / 5331 000.010
Thermomixer comfort	Eppendorf	5355 000.011

PCR for sequence verification and recovery:

- Big Dye Terminator v3.1 Cycle Sequencing Kit, Applied Biosystems, USA, Part. No. 4336917
- DyeEx 2.0 Spin Kit, Qiagen, Germany, Catalogue # 63206
- Finnzyme, Phusion High Fidelity PCR Kit, New England Biolabs, # F-553

Description / Name	Supplier	Cat.No.
Mastercycler / Mastercycler gradient	Eppendorf	5333 000.018 / 5331 000.010

Transformation of the microalga:

- Microcon Centrifugal Filter Devices, Ultracell YM-30, Millipore, Germany, Catalogue # 42410
- 100 % Ethanol

Description / Name	Supplier	Cat.No.
Spermidine	Sigma	85558
TOP-Mix 95323 Bioblock Scientific (Vortexer)	Heidolph Instruments	
Biolistic PDS-1000/He, Particle Delivery System	BioRad	165-2257

Sequence Alignment

The alignment of the PnpUC vector sequence with sequencing results of isolated vector DNA from single *E. coli* clones is shown in the following.

Due to the assembly of the MCS in the vector and hence sequence direction, the alignment is shown in reverse complement direction. Underlined sequences depict M13 forward and reverse primers; italic letters in red show the *neo* gene sequence; italic letters in blue represent the putative *fcp* promoter region. Bold letters in the neo gene section represent start (CAT, *i.e.* ATG) and end codon (TTA, *i.e.* TAA) in reverse complement direction. One basepair mismatch at position 1277 of the vector occurring in every clone is stressed as bold and pink letter.

CLUSTAL 2.0.11 multiple sequence alignment

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_1_comp1ete      -----CTCTAGATTTTGAAGAA 17
_10_comp1ete    -----CTCTAGATTTTGAAGAA 17
_6_comp1ete     -----CTCTAGATTTTGAAGAA 17
_5_comp1ete     -----ACTCTAGATTTTGAAGAA 18
_8_comp1ete     -----ACTCTAGATTTTGAAGAA 18
_9_comp1ete     -----ACTCTAGATTTTGAAGAA 18
_3_comp1ete     -----CTCTAGATTTTGAAGAA 17
PnpUC18        -----GTAAACGACGGCCAGTGCCAAGCTTGCAATGCCCTGCAGGTCGACTCTAGATTTTGAAGAA 60
_2_comp1ete     -----AGATTTTGAAGAA 13
_7_comp1ete     -----TTTGAAGAA 10
                *****

_1_comp1ete     CTCATCAAGAAAGTCTGTAGAAGGCAATTCTCTGGGAGTCAAGG-GCTGCAATGCCATAGA 76
_10_comp1ete    CTCATCAAGAAAGTCTGTAGAAGGCAATTCTCTGGGAGTCAAGG-GCTGCAATGCCATAGA 76
_6_comp1ete     CTCATCAAGAAAGTCTGTAGAAGGCAATTCTCTGGGAGTCAAGG-GCTGCAATGCCATAGA 76
_5_comp1ete     CTCATCAAGAAAGTCTGTAGAAGGCAATTCTCTGGGAGTCAAGG-GCTGCAATGCCATAGA 77
_8_comp1ete     CTCATCAAGAAAGTCTGTAGAAGGCAATTCTCTGGGAGTCAAGG-GCTGCAATGCCATAGA 77
_9_comp1ete     CTCATCAAGAAAGTCTGTAGAAGGCAATTCTCTGGGAGTCAAGG-GCTGCAATGCCATAGA 76
_3_comp1ete     PnpUC18
                CTCATCAAGAAAGTCTGTAGAAGGCAATTCTCTGGGAGTCAAGG-GCTGCAATGCCATAGA 73
_2_comp1ete     CTCATCAAGAAAGTCTGTAGAAGGCAATTCTCTGGGAGTCAAGG-GCTGCAATGCCATAGA 69
_7_comp1ete     *****

_1_comp1ete     GCACGTAGGAACCTGTCTGCCCACTCTCCCCCTAGAGCTCTTCTGCTATATGTCCTGGTTGCTA 136
_10_comp1ete    GCACGTAGGAACCTGTCTGCCCACTCTCCCCCTAGAGCTCTTCTGCTATATGTCCTGGTTGCTA 136
_6_comp1ete     GCACGTAGGAACCTGTCTGCCCACTCTCCCCCTAGAGCTCTTCTGCTATATGTCCTGGTTGCTA 136
_5_comp1ete     GCACGTAGGAACCTGTCTGCCCACTCTCCCCCTAGAGCTCTTCTGCTATATGTCCTGGTTGCTA 137
_8_comp1ete     GCACGTAGGAACCTGTCTGCCCACTCTCCCCCTAGAGCTCTTCTGCTATATGTCCTGGTTGCTA 137
_9_comp1ete     GCACGTAGGAACCTGTCTGCCCACTCTCCCCCTAGAGCTCTTCTGCTATATGTCCTGGTTGCTA 137
_3_comp1ete     GCACGTAGGAACCTGTCTGCCCACTCTCCCCCTAGAGCTCTTCTGCTATATGTCCTGGTTGCTA 136
PnpUC18        GCACGTAGGAACCTGTCTGCCCACTCTCCCCCTAGAGCTCTTCTGCTATATGTCCTGGTTGCTA 179

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_2_comp1ete      GCACCTTAGGAACCTTGTCTTGCCCACTTCCCCCTTAGGCTTCTTGCTATGTCCCTGGTTGCTTA      133
_7_comp1ete      GCACCTTAGGAACCTTGTCTTGCCCACTTCCCCCTTAGGCTTCTTGCTATGTCCCTGGTTGCTTA      129
*****

_1_comp1ete      GGGCAATGTCCTGGTACCTGTGTCAAGCCACTTCCCAAGCCCTTGGCCAAGTCTATGTAAAGCCAGAGA      196
_10_comp1ete     GGGCAATGTCCTGGTACCTGTGTCAAGCCACTTCCCAAGCCCTTGGCCAAGTCTATGTAAAGCCAGAGA      196
_6_comp1ete      GGGCAATGTCCTGGTACCTGTGTCAAGCCACTTCCCAAGCCCTTGGCCAAGTCTATGTAAAGCCAGAGA      196
_5_comp1ete      GGGCAATGTCCTGGTACCTGTGTCAAGCCACTTCCCAAGCCCTTGGCCAAGTCTATGTAAAGCCAGAGA      197
_8_comp1ete      GGGCAATGTCCTGGTACCTGTGTCAAGCCACTTCCCAAGCCCTTGGCCAAGTCTATGTAAAGCCAGAGA      197
_9_comp1ete      GGGCAATGTCCTGGTACCTGTGTCAAGCCACTTCCCAAGCCCTTGGCCAAGTCTATGTAAAGCCAGAGA      196
_3_comp1ete      GGGCAATGTCCTGGTACCTGTGTCAAGCCACTTCCCAAGCCCTTGGCCAAGTCTATGTAAAGCCAGAGA      193
PnpUC18          GGGCAATGTCCTGGTACCTGTGTCAAGCCACTTCCCAAGCCCTTGGCCAAGTCTATGTAAAGCCAGAGA      189
*****

_2_comp1ete      ACCTTCCATTTTCAACCATGATGTTGGGAAAGGCGAAGCCATCCCATGATGTCACCACTAAGGT      256
_10_comp1ete     ACCTTCCATTTTCAACCATGATGTTGGGAAAGGCGAAGCCATCCCATGATGTCACCACTAAGGT      256
_6_comp1ete      ACCTTCCATTTTCAACCATGATGTTGGGAAAGGCGAAGCCATCCCATGATGTCACCACTAAGGT      256
_5_comp1ete      ACCTTCCATTTTCAACCATGATGTTGGGAAAGGCGAAGCCATCCCATGATGTCACCACTAAGGT      257
_8_comp1ete      ACCTTCCATTTTCAACCATGATGTTGGGAAAGGCGAAGCCATCCCATGATGTCACCACTAAGGT      257
_9_comp1ete      ACCTTCCATTTTCAACCATGATGTTGGGAAAGGCGAAGCCATCCCATGATGTCACCACTAAGGT      256
_3_comp1ete      ACCTTCCATTTTCAACCATGATGTTGGGAAAGGCGAAGCCATCCCATGATGTCACCACTAAGGT      253
PnpUC18          ACCTTCCATTTTCAACCATGATGTTGGGAAAGGCGAAGCCATCCCATGATGTCACCACTAAGGT      249
*****

_2_comp1ete      ACCTTCCATTTTCAACCATGATGTTGGGAAAGGCGAAGCCATCCCATGATGTCACCACTAAGGT      253
_7_comp1ete      ACCTTCCATTTTCAACCATGATGTTGGGAAAGGCGAAGCCATCCCATGATGTCACCACTAAGGT      249
*****

_1_comp1ete      CCTCACCATCTGGCATGATGCCCTTGAAGCCCTGGCAAAATAGTTTCAGCAGGGGCCAAGGCCCT      316
_10_comp1ete     CCTCACCATCTGGCATGATGCCCTTGAAGCCCTGGCAAAATAGTTTCAGCAGGGGCCAAGGCCCT      316
_6_comp1ete      CCTCACCATCTGGCATGATGCCCTTGAAGCCCTGGCAAAATAGTTTCAGCAGGGGCCAAGGCCCT      316
_5_comp1ete      CCTCACCATCTGGCATGATGCCCTTGAAGCCCTGGCAAAATAGTTTCAGCAGGGGCCAAGGCCCT      317
_8_comp1ete      CCTCACCATCTGGCATGATGCCCTTGAAGCCCTGGCAAAATAGTTTCAGCAGGGGCCAAGGCCCT      317
_9_comp1ete      CCTCACCATCTGGCATGATGCCCTTGAAGCCCTGGCAAAATAGTTTCAGCAGGGGCCAAGGCCCT      317
_3_comp1ete      CCTCACCATCTGGCATGATGCCCTTGAAGCCCTGGCAAAATAGTTTCAGCAGGGGCCAAGGCCCT      316

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_3_comp1ete      GTGGAGTCTTCTCATGGCAT----- 451
PnpUC18         GTGGAGTCTTCTCATGGCAT----- 494
_2_comp1ete     GTGGAGTCTTCTCATGGCAT----- 448
_7_comp1ete     GTGGAGTCTTCTCATGGCAT----- 444
*****

_1_comp1ete     -----CAGCCATGATTTGACACTTTTCTCAGCTGGAGCTTAGGTTGAGAGAAAGGAGG 501
_10_comp1ete    TGCCAACCATCAGCCATGATTTGACACTTTTCTCAGCTGGAGCTTAGGTTGAGAGAAAGGAGG 550
_6_comp1ete     -----CAGCCATGATTTGACACTTTTCTCAGCTGGAGCTTAGGTTGAGAGAAAGGAGG 501
_5_comp1ete     -----CAGCCATGATTTGACACTTTTCTCAGCTGGAGCTTAGGTTGAGAGAAAGGAGG 502
_8_comp1ete     -----CAGCCATGATTTGACACTTTTCTCAGCTGGAGCTTAGGTTGAGAGAAAGGAGG 502
_9_comp1ete     -----CAGCCATGATTTGACACTTTTCTCAGCTGGAGCTTAGGTTGAGAGAAAGGAGG 502
_3_comp1ete     -----CAGCCATGATTTGACACTTTTCTCAGCTGGAGCTTAGGTTGAGAGAAAGGAGG 501
PnpUC18         -----CAGCCATGATTTGACACTTTTCTCAGCTGGAGCTTAGGTTGAGAGAAAGGAGG 544
_2_comp1ete     -----CAGCCATGATTTGACACTTTTCTCAGCTGGAGCTTAGGTTGAGAGAAAGGAGG 498
_7_comp1ete     -----CAGCCATGATTTGACACTTTTCTCAGCTGGAGCTTAGGTTGAGAGAAAGGAGG 494
*****

_1_comp1ete     TCCTGGCCAGGCACTTCACTTAGTAGGAGGCCAGTCCCTTCCAGCTTCTGTGACCAATCA 561
_10_comp1ete    TCCTGGCCAGGCACTTCACTTAGTAGGAGGCCAGTCCCTTCCAGCTTCTGTGACCAATCA 610
_6_comp1ete     TCCTGGCCAGGCACTTCACTTAGTAGGAGGCCAGTCCCTTCCAGCTTCTGTGACCAATCA 561
_5_comp1ete     TCCTGGCCAGGCACTTCACTTAGTAGGAGGCCAGTCCCTTCCAGCTTCTGTGACCAATCA 562
_8_comp1ete     TCCTGGCCAGGCACTTCACTTAGTAGGAGGCCAGTCCCTTCCAGCTTCTGTGACCAATCA 562
_9_comp1ete     TCCTGGCCAGGCACTTCACTTAGTAGGAGGCCAGTCCCTTCCAGCTTCTGTGACCAATCA 561
_3_comp1ete     TCCTGGCCAGGCACTTCACTTAGTAGGAGGCCAGTCCCTTCCAGCTTCTGTGACCAATCA 604
PnpUC18         TCCTGGCCAGGCACTTCACTTAGTAGGAGGCCAGTCCCTTCCAGCTTCTGTGACCAATCA 558
_2_comp1ete     TCCTGGCCAGGCACTTCACTTAGTAGGAGGCCAGTCCCTTCCAGCTTCTGTGACCAATCA 554
_7_comp1ete     *****
*****

_1_comp1ete     AGGACAGCTTGCACAGGGGACCCC-AGTTTGTGGCAAACAAGAGAGTCTGGCAGCCTCATC 620
_10_comp1ete    AGGACAGCTTGCACAGGGGACCCC-AGTTTGTGGCAAACAAGAGAGTCTGGCAGCCTCATC 669
_6_comp1ete     AGGACAGCTTGCACAGGGGACCCC-AGTTTGTGGCAAACAAGAGAGTCTGGCAGCCTCATC 620
_5_comp1ete     AGGACAGCTTGCACAGGGGACCCC-AGTTTGTGGCAAACAAGAGAGTCTGGCAGCCTCATC 621
_8_comp1ete     AGGACAGCTTGCACAGGGGACCCC-AGTTTGTGGCAAACAAGAGAGTCTGGCAGCCTCATC 621

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_9_comp1ete      AGGACAGCTTGCACAGGGGACCCC-AGTTTGTTCGCAACCAAGAGATCTGGCAGCCTCATC 621
_3_comp1ete      AGGACAGCTTGCACAGGGGACCCC-AGTTTGTTCGCAACCAAGAGATCTGGCAGCCTCATC 620
PnpUC18          AGGACAGCTTGCACAGGGGACCCC-AGTTTGTTCGCAACCAAGAGATCTGGCAGCCTCATC 663
_2_comp1ete      AGGACAGCTTGCACAGGGGACCCC-AGTTTGTTCGCAACCAAGAGATCTGGCAGCCTCATC 617
_7_comp1ete      AGGACAGCTTGCACAGGGGACCCC-AGTTTGTTCGCAACCAAGAGATCTGGCAGCCTCATC 613
*****

_1_comp1ete      CTGGAGCTCATTGAGAGCCCACTGAGGTCGTCTTTACAAAAGACTGGCCCTGCTTG 680
_10_comp1ete     CTGGAGCTCATTGAGAGCCCACTGAGGTCGTCTTTACAAAAGACTGGCCCTGCTTG 729
_6_comp1ete      CTGGAGCTCATTGAGAGCCCACTGAGGTCGTCTTTACAAAAGACTGGCCCTGCTTG 680
_5_comp1ete      CTGGAGCTCATTGAGAGCCCACTGAGGTCGTCTTTACAAAAGACTGGCCCTGCTTG 681
_8_comp1ete      CTGGAGCTCATTGAGAGCCCACTGAGGTCGTCTTTACAAAAGACTGGCCCTGCTTG 681
_9_comp1ete      CTGGAGCTCATTGAGAGCCCACTGAGGTCGTCTTTACAAAAGACTGGCCCTGCTTG 681
_3_comp1ete      CTGGAGCTCATTGAGAGCCCACTGAGGTCGTCTTTACAAAAGACTGGCCCTGCTTG 680
PnpUC18          CTGGAGCTCATTGAGAGCCCACTGAGGTCGTCTTTACAAAAGACTGGCCCTGCTTG 723
_2_comp1ete      CTGGAGCTCATTGAGAGCCCACTGAGGTCGTCTTTACAAAAGACTGGCCCTGCTTG 677
_7_comp1ete      CTGGAGCTCATTGAGAGCCCACTGAGGTCGTCTTTACAAAAGACTGGCCCTGCTTG 673
*****

_1_comp1ete      GGCCTGAAAAGTCTGAAAACCTGCTGCATCAGAGCAACCAATGGTCTGTGCCAGTCAAT 740
_10_comp1ete     GGCCTGAAAAGTCTGAAAACCTGCTGCATCAGAGCAACCAATGGTCTGTGCCAGTCAAT 789
_6_comp1ete      GGCCTGAAAAGTCTGAAAACCTGCTGCATCAGAGCAACCAATGGTCTGTGCCAGTCAAT 740
_5_comp1ete      GGCCTGAAAAGTCTGAAAACCTGCTGCATCAGAGCAACCAATGGTCTGTGCCAGTCAAT 741
_8_comp1ete      GGCCTGAAAAGTCTGAAAACCTGCTGCATCAGAGCAACCAATGGTCTGTGCCAGTCAAT 741
_9_comp1ete      GGCCTGAAAAGTCTGAAAACCTGCTGCATCAGAGCAACCAATGGTCTGTGCCAGTCAAT 741
_3_comp1ete      GGCCTGAAAAGTCTGAAAACCTGCTGCATCAGAGCAACCAATGGTCTGTGCCAGTCAAT 740
PnpUC18          GGCCTGAAAAGTCTGAAAACCTGCTGCATCAGAGCAACCAATGGTCTGTGCCAGTCAAT 783
_2_comp1ete      GGCCTGAAAAGTCTGAAAACCTGCTGCATCAGAGCAACCAATGGTCTGTGCCAGTCAAT 737
_7_comp1ete      GGCCTGAAAAGTCTGAAAACCTGCTGCATCAGAGCAACCAATGGTCTGTGCCAGTCAAT 733
*****

_1_comp1ete      GCCAAACAGTCTCTCAACCCAGGAGGCTGGAACCTGCATGTAGGCCATCTTGTTCAAT 800
_10_comp1ete     GCCAAACAGTCTCTCAACCCAGGAGGCTGGAACCTGCATGTAGGCCATCTTGTTCAAT 849
_6_comp1ete      GCCAAACAGTCTCTCAACCCAGGAGGCTGGAACCTGCATGTAGGCCATCTTGTTCAAT 800
_5_comp1ete      GCCAAACAGTCTCTCAACCCAGGAGGCTGGAACCTGCATGTAGGCCATCTTGTTCAAT 801

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_8_comp1ete      GCCAAACAGTCTTCTCAACCCGACGGCAGCTTGGAAACCTTGCATGTAGGCCATCTTGTTC AAT  801
_9_comp1ete      GCCAAACAGTCTTCTCAACCCGACGGCAGCTTGGAAACCTTGCATGTAGGCCATCTTGTTC AAT  801
_3_comp1ete      GCCAAACAGTCTTCTCAACCCGACGGCAGCTTGGAAACCTTGCATGTAGGCCATCTTGTTC AAT  800
PnpUC18          GCCAAACAGTCTTCTCAACCCGACGGCAGCTTGGAAACCTTGCATGTAGGCCATCTTGTTC AAT  843
_2_comp1ete      GCCAAACAGTCTTCTCAACCCGACGGCAGCTTGGAAACCTTGCATGTAGGCCATCTTGTTC AAT  797
_7_comp1ete      GCCAAACAGTCTTCTCAACCCGACGGCAGCTTGGAAACCTTGCATGTAGGCCATCTTGTTC AAT  793
*****

_1_comp1ete      CATGATGGCCCTCCCTATATAGTGAATCCCGGGGTACCGAGCTCGGTGAGGAAGGAGAGTGA  860
_10_comp1ete     CATGATGGCCCTCCCTATATAGTGAATCCCGGGGTACCGAGCTCGGTGAGGAAGGAGAGTGA  909
_6_comp1ete      CATGATGGCCCTCCCTATATAGTGAATCCCGGGGTACCGAGCTCGGTGAGGAAGGAGAGTGA  860
_5_comp1ete      CATGATGGCCCTCCCTATATAGTGAATCCCGGGGTACCGAGCTCGGTGAGGAAGGAGAGTGA  861
_8_comp1ete      CATGATGGCCCTCCCTATATAGTGAATCCCGGGGTACCGAGCTCGGTGAGGAAGGAGAGTGA  861
_9_comp1ete      CATGATGGCCCTCCCTATATAGTGAATCCCGGGGTACCGAGCTCGGTGAGGAAGGAGAGTGA  861
_3_comp1ete      CATGATGGCCCTCCCTATATAGTGAATCCCGGGGTACCGAGCTCGGTGAGGAAGGAGAGTGA  860
PnpUC18          CATGATGGCCCTCCCTATATAGTGAATCCCGGGGTACCGAGCTCGGTGAGGAAGGAGAGTGA  903
_2_comp1ete      CATGATGGCCCTCCCTATATAGTGAATCCCGGGGTACCGAGCTCGGTGAGGAAGGAGAGTGA  857
_7_comp1ete      CATGATGGCCCTCCCTATATAGTGAATCCCGGGGTACCGAGCTCGGTGAGGAAGGAGAGTGA  853
*****

_1_comp1ete      TGATGCTTTCGTGTGACGCTTACTGTGCTGATTCCTCGACATTT-CTCGACTGGCCAAACACGA  919
_10_comp1ete     TGATGCTTTCGTGTGACGCTTACTGTGCTGATTCCTCGACATTT-CTCGACTGGCCAAACACGA  968
_6_comp1ete      TGATGCTTTCGTGTGACGCTTACTGTGCTGATTCCTCGACATTT-CTCGACTGGCCAAACACGA  919
_5_comp1ete      TGATGCTTTCGTGTGACGCTTACTGTGCTGATTCCTCGACATTT-CTCGACTGGCCAAACACGA  920
_8_comp1ete      TGATGCTTTCGTGTGACGCTTACTGTGCTGATTCCTCGACATTT-CTCGACTGGCCAAACACGA  920
_9_comp1ete      TGATGCTTTCGTGTGACGCTTACTGTGCTGATTCCTCGACATTT-CTCGACTGGCCAAACACGA  920
_3_comp1ete      TGATGCTTTCGTGTGACGCTTACTGTGCTGATTCCTCGACATTT-CTCGACTGGCCAAACACGA  919
PnpUC18          TGATGCTTTCGTGTGACGCTTACTGTGCTGATTCCTCGACATTT-CTCGACTGGCCAAACACGA  962
_2_comp1ete      TGATGCTTTCGTGTGACGCTTACTGTGCTGATTCCTCGACATTT-CTCGACTGGCCAAACACGA  916
_7_comp1ete      TGATGCTTTCGTGTGACGCTTACTGTGCTGATTCCTCGACATTT-CTCGACTGGCCAAACACGA  912
*****

_1_comp1ete      AGTGTGCGGATCGGG-TTGGCCC-ATTTT-----CTTTTTTCCCTTTCT-----TTTTTT-----GGG  968
_10_comp1ete     AGTGTGCGGATCGGG-TTGGCCC-ATTTT-----CTTTTTTCCCTTTCT-----TTTTTT-----GGG  1017

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_6_comp1ete      AGTGCCTGCCGATCGGG-TTTGGCCC-ATTTT---CTTTTTTTCCTTTCT---TTTTTTT---GGG 968
_5_comp1ete      AGTGCCTGCCGATCGGG-TTTGGCCC-ATTTT---CTTTTTTTCCTTTCT---TTTTTTT---GGG 969
_8_comp1ete      AGTGCCTGCCGATCGGG-TTTGGCCC-ATTTT---CTTTTTTTCCTTTCT---TTTTTTT---GGG 969
_9_comp1ete      AGTGCCTGCCGATCGGG-TTTGGCCC-ATTTT---CTTTTTTTCCTTTCT---TTTTTTT---GGG 969
_3_comp1ete      AGTGCCTGCCGATCGGG-TTTGGCCC-ATTTT---CTTTTTTTCCTTTCT---TTTTTTT---GGG 968
PnpUC18          AGTGCCTGCCGATCGGG-TTTGGCCC-ATTTT---CTTTTTTTCCTTTCT---TTTTTTT---GGG 1011
_2_comp1ete      AGTGCCTGCCGATCGGG-TTTGGCCC-ATTTT---CTTTTTTTCCTTTCT---TTTTTTT---GGG 965
_7_comp1ete      AGTGCCTGCCGATCGGG-TTTGGCCC-ATTTT---CTTTTTTTCCTTTCT---TTTTTTT---GGG 961
*****          *****          *****          *****          *****          *****
_1_comp1ete      CCCTGTGGG-CACCCCTAACCAAGCTAAGCCCAACGCGAGCATAGCTGTGAAGTGTGCGGA 1027
_10_comp1ete     CCCTGTGGG-CACCCCTAACCAAGCTAAGCCCAACGCGAGCATAGCTGTGAAGTGTGCGGA 1076
_6_comp1ete      CCCTGTGGG-CACCCCTAACCAAGCTAAGCCCAACGCGAGCATAGCTGTGAAGTGTGCGGA 1027
_5_comp1ete      CCCTGTGGG-CACCCCTAACCAAGCTAAGCCCAACGCGAGCATAGCTGTGAAGTGTGCGGA 1028
_8_comp1ete      CCCTGTGGG-CACCCCTAACCAAGCTAAGCCCAACGCGAGCATAGCTGTGAAGTGTGCGGA 1028
_9_comp1ete      CCCTGTGGG-CACCCCTAACCAAGCTAAGCCCAACGCGAGCATAGCTGTGAAGTGTGCGGA 1028
_3_comp1ete      CCCTGTGGG-CACCCCTAACCAAGCTAAGCCCAACGCGAGCATAGCTGTGAAGTGTGCGGA 1027
PnpUC18          CCCTGTGGG-CACCCCTAACCAAGCTAAGCCCAACGCGAGCATAGCTGTGAAGTGTGCGGA 1070
_2_comp1ete      CCCTGTGGG-CACCCCTAACCAAGCTAAGCCCAACGCGAGCATAGCTGTGAAGTGTGCGGA 1024
_7_comp1ete      CCCTGTGGG-CACCCCTAACCAAGCTAAGCCCAACGCGAGCATAGCTGTGAAGTGTGCGGA 1020
*****          *****          *****          *****          *****          *****
_1_comp1ete      ACTGGGAGTTTACCTTTGTGCAAGGGCGTGAAGCTGCGCCGCGAACCCCGAGGCGTGAACCATCG 1087
_10_comp1ete     ACTGGGAGTTTACCTTTGTGCAAGGGCGTGAAGCTGCGCCGCGAACCCCGAGGCGTGAACCATCG 1136
_6_comp1ete      ACTGGGAGTTTACCTTTGTGCAAGGGCGTGAAGCTGCGCCGCGAACCCCGAGGCGTGAACCATCG 1087
_5_comp1ete      ACTGGGAGTTTACCTTTGTGCAAGGGCGTGAAGCTGCGCCGCGAACCCCGAGGCGTGAACCATCG 1088
_8_comp1ete      ACTGGGAGTTTACCTTTGTGCAAGGGCGTGAAGCTGCGCCGCGAACCCCGAGGCGTGAACCATCG 1088
_9_comp1ete      ACTGGGAGTTTACCTTTGTGCAAGGGCGTGAAGCTGCGCCGCGAACCCCGAGGCGTGAACCATCG 1088
_3_comp1ete      ACTGGGAGTTTACCTTTGTGCAAGGGCGTGAAGCTGCGCCGCGAACCCCGAGGCGTGAACCATCG 1087
PnpUC18          ACTGGGAGTTTACCTTTGTGCAAGGGCGTGAAGCTGCGCCGCGAACCCCGAGGCGTGAACCATCG 1130
_2_comp1ete      ACTGGGAGTTTACCTTTGTGCAAGGGCGTGAAGCTGCGCCGCGAACCCCGAGGCGTGAACCATCG 1084
_7_comp1ete      ACTGGGAGTTTACCTTTGTGCAAGGGCGTGAAGCTGCGCCGCGAACCCCGAGGCGTGAACCATCG 1080
*****          *****          *****          *****          *****          *****
_1_comp1ete      CCTTTGGGCCCGTGAACCGCGAATATGCAAGCGTGTCTGCAAGCCTTTTGAACGCAAGTCCGCAA 1147

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_10_comp1ete      CCTTTGGGCCCGTTGAACCCGGCCGATATGCAAGCCGTGTTCTTGCAGCCCTTTTGAACCGCAGGTCGCCAA 1196
_6_comp1ete      CCTTTGGGCCCGTTGAACCCGGCCGATATGCAAGCCGTGTTCTTGCAGCCCTTTTGAACCGCAGGTCGCCAA 1147
_5_comp1ete      CCTTTGGGCCCGTTGAACCCGGCCGATATGCAAGCCGTGTTCTTGCAAGCCCTTTTGAACCGCAGGTCGCCAA 1148
_8_comp1ete      CCTTTGGGCCCGTTGAACCCGGCCGATATGCAAGCCGTGTTCTTGCAAGCCCTTTTGAACCGCAGGTCGCCAA 1148
_9_comp1ete      CCTTTGGGCCCGTTGAACCCGGCCGATATGCAAGCCGTGTTCTTGCAAGCCCTTTTGAACCGCAGGTCGCCAA 1147
_3_comp1ete      CCTTTGGGCCCGTTGAACCCGGCCGATATGCAAGCCGTGTTCTTGCAAGCCCTTTTGAACCGCAGGTCGCCAA 1190
PnpUC18          CCTTTGGGCCCGTTGAACCCGGCCGATATGCAAGCCGTGTTCTTGCAAGCCCTTTTGAACCGCAGGTCGCCAA 1144
_2_comp1ete      CCTTTGGGCCCGTTGAACCCGGCCGATATGCAAGCCGTGTTCTTGCAAGCCCTTTTGAACCGCAGGTCGCCAA 1144
_7_comp1ete      CCTTTGGGCCCGTTGAACCCGGCCGATATGCAAGCCGTGTTCTTGCAAGCCCTTTTGAACCGCAGGTCGCCAA 1140
*****

_1_comp1ete      GAAAGAGCGACTGCCACTCCCGCCGGCCCGCCCTTGCGGAGCCGGCCGATGCGCCCGACGCGC 1207
_10_comp1ete     GAAAGAGCGACTGCCACTCCCGCCGGCCCGCCCTTGCGGAGCCGGCCGATGCGCCCGACGCGC 1256
_6_comp1ete      GAAAGAGCGACTGCCACTCCCGCCGGCCCGCCCTTGCGGAGCCGGCCGATGCGCCCGACGCGC 1207
_5_comp1ete      GAAAGAGCGACTGCCACTCCCGCCGGCCCGCCCTTGCGGAGCCGGCCGATGCGCCCGACGCGC 1208
_8_comp1ete      GAAAGAGCGACTGCCACTCCCGCCGGCCCGCCCTTGCGGAGCCGGCCGATGCGCCCGACGCGC 1208
_9_comp1ete      GAAAGAGCGACTGCCACTCCCGCCGGCCCGCCCTTGCGGAGCCGGCCGATGCGCCCGACGCGC 1208
_3_comp1ete      GAAAGAGCGACTGCCACTCCCGCCGGCCCGCCCTTGCGGAGCCGGCCGATGCGCCCGACGCGC 1207
PnpUC18          GAAAGAGCGACTGCCACTCCCGCCGGCCCGCCCTTGCGGAGCCGGCCGATGCGCCCGACGCGC 1250
_2_comp1ete      GAAAGAGCGACTGCCACTCCCGCCGGCCCGCCCTTGCGGAGCCGGCCGATGCGCCCGACGCGC 1204
_7_comp1ete      GAAAGAGCGACTGCCACTCCCGCCGGCCCGCCCTTGCGGAGCCGGCCGATGCGCCCGACGCGC 1200
*****

_1_comp1ete      TCTTGCCCATGACCTTCCGGGAGAGGTTTGAAGCCGGCCGACCTTCGGTGAAGGTTTACTTTAAGTTTCGTTCT 1267
_10_comp1ete     TCTTGCCCATGACCTTCCGGGAGAGGTTTGAAGCCGGCCGACCTTCGGTGAAGGTTTACTTTAAGTTTCGTTCT 1316
_6_comp1ete      TCTTGCCCATGACCTTCCGGGAGAGGTTTGAAGCCGGCCGACCTTCGGTGAAGGTTTACTTTAAGTTTCGTTCT 1267
_5_comp1ete      TCTTGCCCATGACCTTCCGGGAGAGGTTTGAAGCCGGCCGACCTTCGGTGAAGGTTTACTTTAAGTTTCGTTCT 1268
_8_comp1ete      TCTTGCCCATGACCTTCCGGGAGAGGTTTGAAGCCGGCCGACCTTCGGTGAAGGTTTACTTTAAGTTTCGTTCT 1268
_9_comp1ete      TCTTGCCCATGACCTTCCGGGAGAGGTTTGAAGCCGGCCGACCTTCGGTGAAGGTTTACTTTAAGTTTCGTTCT 1268
_3_comp1ete      TCTTGCCCATGACCTTCCGGGAGAGGTTTGAAGCCGGCCGACCTTCGGTGAAGGTTTACTTTAAGTTTCGTTCT 1267
PnpUC18          TCTTGCCCATGACCTTCCGGGAGAGGTTTGAAGCCGGCCGACCTTCGGTGAAGGTTTACTTTAAGTTTCGTTCT 1310
_2_comp1ete      TCTTGCCCATGACCTTCCGGGAGAGGTTTGAAGCCGGCCGACCTTCGGTGAAGGTTTACTTTAAGTTTCGTTCT 1264
_7_comp1ete      TCTTGCCCATGACCTTCCGGGAGAGGTTTGAAGCCGGCCGACCTTCGGTGAAGGTTTACTTTAAGTTTCGTTCT 1260
*****

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_1_comp1ete      ACGGAGTGAACAGCACCTTCCGGGGCGGTTTTAGAACTTTGA----- 1306
_10_comp1ete    ACGGAGTGAACAGCACCTTCCGGGGCGGTTTTAGAACTTTGAT----- 1356
_6_comp1ete     ACGGAGTGAACAGCACCTTCCGGGGCGGTTTTAGAACTTTGATTT----- 1308
_5_comp1ete     ACGGAGTGAACAGCACCTTCCGGGGCGGTTTTAGAACTTTGAT----- 1308
_8_comp1ete     ACGGAGTGAACAGCACCTTCCGGGGCGGTTTTAGAACTTTGAT----- 1307
_9_comp1ete     ACGGAGTGAACAGCACCTTCCGGGGCGGTTTTAGAACTTTGAT----- 1307
_3_comp1ete     ACGGAGTGAACAGCACCTTCCGGGGCGGTTTTAGAACTTTGAT----- 1308
PnpUC18         ACGGAGTGAACAGCACCTTCCGGGGCGGTTTTAGAACTTTGATTTGATG----- 1370
_2_comp1ete     ACGGAGTGAACAGCACCTTCCGGGGCGGTTTTAGAACTTTGATTTG----- 1306
_7_comp1ete     ACGGAGTGAACAGCACCTTCCGGGGCGGTTTTAGAACTTTGAT----- 1299
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_1_comp1ete      -----
_10_comp1ete    -----
_6_comp1ete     -----
_5_comp1ete     -----
_8_comp1ete     -----
_9_comp1ete     -----
_3_comp1ete     -----
PnpUC18         AATTCTTAATCATGTGTCATAGCTGTTTCTGTGTG 1404
_2_comp1ete     -----
_7_comp1ete     -----

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