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Conjugative DNA transfer between bacteria and the heterokont alga Nannochloropsis

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Abstract

The heterokont microalgae *Nannochloropsis* have been the subject of many studies that focus on genomic and metabolic engineering. Research on this genus has been motivated by the algae's high-level production of lipids and in particular polyunsaturated fatty acids. The work presented in this thesis is part of the effort to develop new tools that enable the rapid and reliable generation of *Nannochloropsis oceanica* mutants.

A new growth medium was developed and evaluated against established *Nannochloropsis* growth media. Compared with previously described growth media, the new medium can be prepared without filtered sea water and improved growth of *N. oceanica*.

Transkingdom conjugation has recently been established as a method for transfer of DNA from *Escherichia coli* to two species of heterokont algae (*Phaeodactylum tricornutum* and *Thalassiosira pseudonana*). For this phylum of algae, conjugation has the potential to replace the standard methods of transformation by particle bombardment and electroporation. This project focuses on the development of transkingdom conjugation as a method of establishing mutants of *N. oceanica* (CCMP1779). Two *E. coli* strains were used as donors in the project, S17-1 which has chromosomally integrated transfer functions, and DH10B harbouring the mobilization plasmid, pTA-Mob. Of these two donor strain, plasmid transfer mediated by DH10B/pTA-Mob resulted in the highest conjugation efficiency.

Preliminary work identified, that co-incubation of the recipient and donor cells at 30 °C was crucial for establishing conjugation. Various conditions and parameters were investigated for their effect on conjugation efficiency. The parameter of recipient to donor ratio cells was found to be an important factor, but with inconclusive results, further research is needed. During co-incubation of recipient and donor cells, dark conditions resulted in higher conjugation efficiencies than when cells were exposed to light. Temporal variations of the co-incubation identified a maximum for conjugation efficiency at around 90 minutes.

A set of novel plasmids, pAPA0602 and pAPA0169, containing endogenous promoters with reported high expression levels in *N. oceanica* and the standard plasmid pSELECT100 were used in the conjugal transfer. ALl three of these base vectors were gentically altered to include the origin of transfer, *oriT*. Two additional constructs were created from the pAPA-plasmids, which included the yeast sequence, *CEN6-ARSH4-HIS3*, that has been reported to enable episomal

plasmid replication in diatoms. Of these five constructs, the highest conjugation efficiency was observed with pAPA0169.

Transformation of *N. oceanica* by electroporation was conducted to compare transformation efficiencies with conjugation efficiencies. The highest transformation efficiency obtained by electroporation was with the plasmid pAPA0602. Electroporation was also used as the method to transform *N. oceanica* with pAPA0602 and pAPA0169, to evaluate the tolerance against zeocin conferred by a zeocin resistance gene under control of the pAPA plasmid-promoters. The plasmids, pAPA0602 and pAPA0169 conferred tolerance to the antibiotic at similar levels. Overall efficiencies obtained for pAPA0169 and pAPA0602 in this project were higher than previously reported numbers.

The optimized conjugation protocol, together with the developed plasmids and improved growth medium for mutant generation, extends the molecular toolbox for *N. oceanica*.

Sammendrag

Den heterokonte mikroalgen *Nannochloropsis* har vært gjenstand for mange forskningsstudier. Forskrningen har vært motivert av algenes høy produksjonsnivå av flerumettede fettsyrer.

Konjugering ble nylig etablert som en vellykket metode for overføring av DNA i to arter av heterokontalger (*P. tricornutum* og *T. pseudonana*). For denne phylum av alger har konjugering et potensial til erstatte standardmetoder for transformasjon som partikkelbombardement og elektroporasjon. Dette prosjektet fokuserer på utvikling av konjugering som en metode for DNA-overføring til *Nannochloropsis oceanica* (CCMP1779). To *Escherichia coli* stammer ble brukt som donorer i prosjektet, S17-1 som har kromosomalt integrerte overføringsfunksjoner, og DH10B som inneholder mobiliseringsplasmidet, pTA-Mob. Av de to donorstammen resulterte plasmidoverføring mediert av DH10B/pTA-Mob i den høyeste konjugeringseffektivitet.

Under arbeidet ble det tydelig at en inkubering av mottaker og donorceller ved 30 °C var avgjørende for å etablere konjugering. Ulike forhold og parametere ble undersøkt for deres effekt på konjugasjonens effektivitet. Ratioen mellom mottaker og donor celler ble funnet til å være en viktig faktor for effektiviteten. Det er derimot nødvendig å etterforske parameteren ytterligere for konkludere noe om den. En evaluaering av lysforhold under inkubering av mottaker og donor celler viste at inkubasjon i mørket resulterte i høyere konjugeringseffektivitet. Temporale variasjoner av inkubasjonen resulterte i en effektivitetstopp på 90 minutter.

Et sett med nye plasmider, pAPA0602 og pAPA0169, inneholdende endogene promotorer med rapporterte høye ekspresjonsnivåer i *N. oceanica* og pSELECT100-plasmidet ble anvendt i konjugeringen. Origin of transfer, *oriT*, ble klonet inn i plasmidene. To ytterligere konstruksjoner ble opprettet fra pAPA-plasmidene, som inkluderte gjærsekvensen, *CEN6-ARSH4-HIS3*, som har blitt rapportert for å muliggjøre episomal replikasjon i diatomer. Av de fem konstruksjonene var den høyeste konjugeringseffektivitet oppnådd med pAPA0169. Mutasjoner ble funnet i den klonede *CEN6-ARSH4-HIS3* sekvensen og konstruksjoner som inkluderte sekvensen resulterte ikke i økt konjugeringseffektivitet.

Transformasjon av *N. oceanica* ved elektroporasjon ble utført for å sammenligne transformasjons- med konjugerings-effektivitet. Den høyeste transformasjonseffektiviten oppnådd ved elektroporasjon var med plasmidet pAPA0602. Elektroporasjon ble også brukt som metode for å transformere *N. oceanica* med

pAPA0602 og pAPA0169, i en evaluering av pAPA-promotorer. Plasmidene ga toleranse mot antibiotikaet, zeocin, på like nivåer. Effektivitet oppnådd for pAPA0169 og pAPA0602 i dette prosjektet er høyere enn tidligere rapportert.

Contents

Part I

Introduction

1 Introduction

Microalgae have been at the center of attention, due to a growing global need to replace depleting fossil fuel sources with biofuel from non-food biomass. One such algae, *Nannochloropsis*, has lately seen intense attempts to establish it as a model organism by placing great effort in studying its characteristics as well as establishing tools to genetically engineer it.

The oleaginous microalgae *Nannochloropsis* is an important organism today for its many favourable characteristics, both ecological and industrial. There is only one established method of transformation for *Nannochloropsis* sp. and further research is needed to explore several methods that will expand the molecular tool set for this promising algae. Until recently it has only been possible to introduce DNA to microalgae such as diatoms via particle bombardment, which also resulted in random integration of the DNA into the genome. However, studies have emerged with exciting new tools for improving both conjugal transfer systems and the establishment of such a method for the diatoms *Phaeodactylum tricornutum* and *Thalassiosira pseudonana*. Novel set of plasmids (pAPA1001) have recently been established, which include stable, high expression promoters that are native to *Nannochloropsis* and have the potential to facilitate high efficiency recombination. These findings serve as inspiration for this thesis to further develop molecular tools for *Nannochloropsis*.

In this thesis, I first detail the importance of the unicellular microalgae *Nannochloropsis*, and review the biotechnological advances that have been made in engineering it. I also describe bacterial and transkingdom conjugation, and related work, before I report my work at establishing a new protocol for transformating *Nannochloropsis*.

1.1 Aims

A novel plasmid delivery system using transkingdom conjugation has been reported for heterokont algae, as an alternative to electroporation and biolistic transformation. The aim of this project is to achieve transkingdom conjugation between *Escherichia coli* and *N. oceanica* CCMP1779 which could establish a tool for easier and more efficient transformation.

In addition, an effort will be made to optimize growth conditions for the algae by evaluating different growth media.

The pAPA1001 plasmids are promising as they contain native Nannochloropsis CCMP1179 promoters that have a higher and more stable level of expression than other reported plasmids [Nguyen, 2016, Kilian et al., 2011, Vieler et al., 2012]. Expression will be reported by a zeocin resistance gene, which has been used before in *Nannochloropsis* and proved to be a reliable selection marker. An attempt will be made to establish a protocol for conjugal transfer of these plasmids, as well as a control plasmid pSELECT100. This will be achieved by first cloning the origin of transfer (*oriT*) into the plasmids and introducing the plasmids to donor strains. Two donor strains will be evaluated: *E. coli* S17-1 with chromosomally integrated transfer functions and *E. coli* DH10B carrying the mobilization plasmid pTA-Mob. Different conditions and parameters for optimization of the protocol will be tested and reported.

The pAPA1001 plasmid will also be altered to include a yeast sequence with the potential to improve efficiency by allowing the recipient to maintain the plasmids as episomes [Karas et al., 2015].

Conjugation efficiencies for all plasmids and constructs will be compared with their electroporation effiencies, following a standard electroporation protocol based on past studies [Vieler et al., 2012, Kilian et al., 2011, Radakovits et al., 2012].

The strength of the endogenous promoters of pAPA1001 will be evaluated by testing the tolerance to zeocin they confer on to *N. oceanica*.

2 Background

In this chapter, background information will be given about *Nannochloropsis*, its genome and established molecular tools. In addition, horizontal gene transfer, focusing on conjugation as well as electroporation will be reviewed as methods of DNA transfer.

2.1 *Nannochloropsis*

Nannochloropsis is an eukaryotic, unicellular microalgae in the Eustigmatophyceae class of the phylum Heterokontophyta. Heterokonts or Stramenopiles compromise a large phylum with around 25 000 species, relating *Nannochloropsis* to brown algae and diatoms. The genus of *Nannochloropsis* has six known species as of today; *N. oceanica*, *N. gaditana*, *N. granulata*, *N. limnetica*, *N. oculata* and *N. salina*. *Nannochloropsis* sp. were first classified by D. J. Hibberd in 1981 and have been identified in both fresh and marine water [Hibberd, 1981]. The algae are usually described as nonmotile, small (∼2-8 µm) , spherical and with no unique morphological characteristics. They are distinguishable by sequence analysis of 18S rDNA or rbcL gene [Andersen et al., 1998, Fawley and Fawley, 2007].

It has been indicated that *N. oceanica* possesses nuclear monoploidy and undergoes asexual propagation, leading to the hypothesis that *N. oceanica* is a premeiotic or ameiotic alga [Pan et al., 2011].

The plastids of *Nannochloropsis* are surrounded by four membranes derived from a secondary endosymbiotic event [Murakami and Hashimoto, 2009]. Its

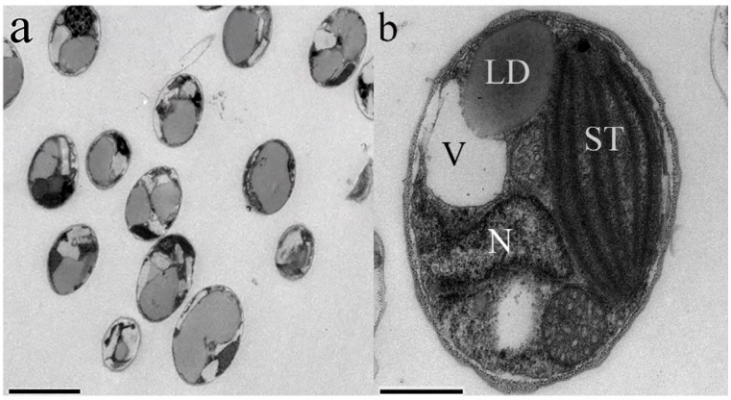


Figure 2.1: *N. oculata* micrographs showing (a) cell morphology and (b) organelle structure. LD, lipid droplet; ST, thylakoid stacks; N, nucelus; V, vacuoles. Bars = 2 μm for (a) and 0.5 μm for (b) [Ma et al., 2016].

chloroplast resembles that of plants and remains close to the nucleus, while a lipid droplet serves as energy storage (Figure 2.1), changing in size under stress conditions [Ma et al., 2016]. *Nannochloropsis* species are characterized by chlorophyll a and lacking both chlorophyll b and c. They possesses other pigments as well, such as β-carotene, violaxanthin and vaucheriaxanthin, as well as the carotenoids canthaxanthin and astaxanthin. In some *Nannochloropsis* species, the relative carotenoid content increases when the organism undergoes nutrient depletion, something that has been considered of commercial value [Lubián et al., 2000].

In general, unicellular algae have in the past years received a gradually increasing interest worldwide from research and industrial institutions. They are known for their irreplaceable role in ecosystems and the environment as primary producers in food chains due their high nutrient content [Cardinale et al., 2011]. Microalgae have also contributed to mitigating the greenhouse effect and water pollution [Walsh et al., 2016]. However, the characteristic that has garnered the most attention is their ability to produce and accumulate high levels of polyunsaturated fatty acids (PUFAs). *Nannochloropsis* has been found to accumulate oil up to 60% of its dry weight under nitrogen-starvation, a very desirable quality for biofuel production [Kilian et al., 2011]. *Nannochloropsis oceanica* has been found to produce the highest lipid content compared to other *Nannochlorpsis*

sp [Beacham et al., 2014]. As algae do not take up land space, can be cultured in wastewater and salt water, and grow rapidly, these features place them in favour over plants as oil crops [Sukarni et al., 2014]. *Nannochloropsis* has become an established industrial alga and has been grown for aquaculture in photobioreactors as well as outdoor ponds [Tomaselli, 2004]. Aside from its potential use for biodiesel, *Nannochloropsis* contains other valuable substances such as high-quality protein, eicosapentaenoic acid (EPA) and ω3 fatty acids, an important dietary supplement [Kagan and Matulka, 2015, Andrés et al., 1992].

2.1.1 Genome of *N. oceanica*

Based on the aforementioned qualities, it is no wonder many studies have and are still being conducted to optimize *Nannochloropsis* growth, to understand under which circumstances it accumulates certain compounds, and how to genetically manipulate it. The 28.7 Mb genome of the *N. oceanica* strain CCMP1779 has been sequenced and revealed that this strain in particular, and perhaps others in general, possess a high gene density and low presence of repetitive DNA [Vieler et al., 2012].

Genome manipulation in *Nannochloropsis* can occur via homologous recombination, where a gene of interest is altered by targeting with a designed complementary sequence and such recombination-based transformation has been established [Kilian et al., 2011]. Another way of stable integration of exogenous DNA in the algae can occur by random insertion. Apart from integration in the nuclear genome, the plastid genome can also be targeted. The effects and lethal concentrations of many antibiotics have also been studied, making it easier to determine suitable selectable markers as well as antibiotics that can be used for maintaining pure cultures [Chernyavskaya, 2014]. In one study the endoplasmic reticulum/secretory pathway, plastid and mitochondria among other cellular compartments were visualized with green fluorescent protein by using specific N-terminal targeting signals as pre-sequences, adding to the molecular tool set of *N. oceanica* [Moog et al., 2015].

2.2 Electroporation

Electroporation is a molecular biology technique used to make recipient cells permeable for uptake of exogenous DNA or other substances. Permeability is achieved by applying an electric current through the cells that disrupts the cell wall and membrane, creating micropores [Weaver and Chizmadzhev, 1996]. For the purposes of transformation, due to the permeability being relatively brief, the genetic material is mixed with the competent cells prior to electroporation. Depending on the desired outcome, transformative DNA can be designed to either remain as a plasmid or integrate into the nuclear or plastid genome.

Several conditions affect the transformation efficiencies when using electroporation, including voltage, amount of DNA, amount of cells and treatment of cells prior and subsequent to application of electric pulses. Electroporation has been applied on bacterial, yeast, plant, mammalian and algal cells for a couple of decades [Chang, 1991].

Recently it has been discovered that electroporation can achieve high efficiency transformation in *Nannochloropsis* sp. [Kilian et al., 2011]. Other studies have confirmed the success of electroporation as a tool for transformation of the algae [Vieler et al., 2012, Radakovits et al., 2012]. In their study, Kilian et al. (2012) revealed that linearization of exogenous DNA is a significant factor for achieving efficient transformation in *Nannochloropsis*, as circular plasmid DNA resulted in almost no transformants. It was also demonstrated that the relationship between the efficiency and amount of DNA used is linear.

Incorporation of the exogenous DNA can sometimes occur randomly or by homologous recombination achieved by including DNA sequences that are homologous to the DNA sequences of *Nannochloropsis*. This was demonstrated, using an endogenous promoter of violaxanthin/chlorophyll a-binding protein (VCP) genes that was used to knockout several genes involved in nitrogen metabolism of *Nannochloropsis* [Kilian et al., 2011]. In another study, expression of resistance conferring gene was achieved using a vector with β-tubulin promoter [Radakovits et al., 2012]. Vieler et al. (2012) reported using the vector pSELECT100 containing the endogenous promoter for lipid droplet surface protein (LDSP) to express a resistance conferring gene in *N. oceanica* CCMP1779 [Vieler et al., 2012]. The transformation efficiencies for these studies range between 1.25 x10^{-5} and 8.3 x 10^{-7} cfu/cells/μg of DNA.

As with any method of transformation, electroporation has its disadvantages. A high voltage can cause damage to the cell's wall beyond its capability for repair. A voltage too low will not result in sufficient permeability of the cell wall and therefore an optimum voltage needs to be found. Cell density and therefore cell wall thickness can also influence the results of electroporation. As cultures become more dense, cells experience stress due to altered nutrient and light conditions, resulting in the formation of thicker cell walls [Beacham et al., 2014].

An important prerequisite to electroporation is the removal of salts. Due to salts being good conductors, the flow of electricity in their presence can destroy the cells. Making cells competent for electroporation therefore involves multiple washing steps. Since *Nannochloropsis* grows in sea salt medium, it is very important to remove any rests by washing with sorbitol solution (Section 7.1).

Only one other study, via agrobacterium-mediated transformation, has shown a way, other than electroporation, of transforming *Nannochloropsis* [Cha et al., 2011]. Cha et al. (2011) used *Nannochloropsis* (strain UMT-M3) and report their findings as a cost efficient transformation protocol for *Nannochloropsis*. However, this method does not allow the transfer of whole plasmids as genes of interest need to be inserted into the T-DNA of the Ti or Ri plasmid. Refer to Section 2.3.3 for more information on *Agrobacterium*.

2.3 Horizontal gene transfer

Genetic transfer occurs naturally both horizontally and vertically. Lateral or horizontal gene transfer (HGT) differs from vertical gene transfer, where genes are passed on from parent cells to offspring and genetic characteristics are inherited in the classical sense. HGT can happen both naturally and artificially between different species.

While the concept of horizontal gene transfer was subjected to scepticism by molecular biologists in the beginning, it eventually became convention and is now considered a cornerstone in evolution [Syvanen and Kado, 2001]. With the development of whole genome sequencing, HGT became more evident as genomes revealed to be strewn with DNA from different species. It has become evident that HGT is prevalent among bacteria and that it drives metabolic evolution. Within a species we can find strains that share a set of core genes, and differ considerably in their variable genes, whose presence is primarily due to HGT or

gene loss [Skippington and Ragan, 2011].

There are several mechanisms for the horizontal transfer of genes and they include; transduction, the transfer of bacterial DNA between a bacteriophage-infected bacterium and a bacteriophage-susceptible bacterium; transformation, the uptake of free DNA by competent bacteria and lastly, conjugation.

Conjugation is defined as the horizontal gene transfer between bacteria and was described in 1946 in an experiment by Joshua Lederberg and Edward Tatum [Tatum and Lederberg, 1947]. Lederberg and Tatum used two strains of *Escherichia coli* where each strain was deficient in producing amino acids and where the deficiencies in between these strains did not overlap. When incubated together and then plated on the minimal media, progeny that possessed wild type properties (being able to produce all amino acids) were found to be present [Griffiths et al., 2000]. In their study, they concluded that the origin of the bacterial mutants in the mixed culture must be the result of hybridization and segregation, as the spontaneous occurrence of such mutations was lower than what occurred when the strains were plated together [Tatum and Lederberg, 1947].

Around the mid-twentieth century, conjugation was pointed out to be the reason of spreading resistance to antimicrobials/antibiotics. The phenomena of antimicrobial resistance and its examination has given more insight into horizontal gene transfer and revealed more information about the species involved. For example, how methicillin-resistant *Staphylococcus aureus* (MRSA) are created, how the resistance genes move and at what rate [Barlow, 2009]. Ways to counteract the growing resistance among microbial populations, especially pathogenic bacteria, is the focus of many studies today. New tools such as CRISPR (Clustered, Regularly Interspaced Short Palindromic Repeats) have been found to counteract routes of HGT and therefore limiting the spread of the resistance by interference with conjugation [Marraffini and Sontheimer, 2008].

2.3.1 Mechanism of conjugation

The most well-known conjugation systems are mediated by plasmids. A conjugal plasmid can belong to one of several incompatibility groups (inc) that determine the relatability of the plasmids and therefore their ability to stably coexist within a cell. There are self-transferable plasmids that encode the necessary functions for their own mobilization and transfer. For this transfer to occur, are three

important loci, *tra* for transfer functions of plasmid, *mob* for mobilization and *oriT*, the origin of transfer have been identified. The *oriT* acts in *cis*, while *mob* and *tra* encode *trans*-acting functions [Heinemann, 1991]. The *trans*-acting genes are involved in DNA metabolism, DNA transport and cell-cell interactions.

A self-transferable plasmid therefore needs all loci to be indeed self-transferable. One example is the fertility (F) factor, carried on the F episome, discovered by Lederberg et al. in the early 1950s [Lederberg et al., 1952]. Strains of *E.coli* K-12 that seemed to have the F factor and were able to act as donors were termed F+. Cells that could not act as donors were therefore named F- cells. This early work was continued by William Hayes, who in 1953 discovered that the F factor can be lost and regained [Griffiths et al., 2000]. In addition to self-transferable plasmids, there are mobilizable plasmids that rely on self-transferable plasmids for transmission. Mobilizable plasmids however must carry the *cis* acting locus *oriT* [Heinemann and Ankenbauer, 1993]. That is because it is at the *oriT* that a nick occurs and the DNA strands are unwound, in preparation for transmission. The nicking and unwinding functions are encoded by the *mob* locus, which encodes plasmid specific functions, while the *tra* genes do not. This means that *mob* genes from different inc groups will not recognize a heterologous *oriT*, but *tra* products can interact with *mob* functions of plasmids of a different inc group [Heinemann, 1991].

The F factor can sometimes also carry one or several insertion-sequences (IS). IS elements are mobile DNA that move within the host chromosome or between chromosomes and plasmids [Griffiths et al., 2000]. Such IS elements can give opportunity to homologous crossovers when they exist in both the plasmid and in the chromosome of the host organism. Naturally, this can lead to the insertion of the conjugative plasmid into the chromosome.

The mechanics of transfer involve cell to cell contact between donor and recipient cells. On the surface of donor cells, there are structures called pili which mediate cell-cell contact. Such an interaction can provide the donor cell with information about the potential recipient, create several more contacts to bring the recipient closer and generate a signal resulting in the preparation of DNA transfer (Figure 2.2) [Heinemann, 1991]. It is thought that a type IV pili are the type that aid the genetic transfer [Madigan et al., 2015]. The pilus is often called the F-pilus or conjugation pilus. After initiating contact, a cellular mating aggregate is formed and stabilized between the cells, and a transport pore extending through the cell walls, connects the cytoplasms of the two cells. After nicking the DNA at *oriT*, the DNA is unwound in rolling-circle mode. A mobilization protein,

Mob, attaches to the 5′-end of the nicked DNA strand and then anchors it by interacting with the transport pore. When the 3′-end of that DNA strand reaches the pore, the ends join, creating a single stranded copy of the plasmid [Yin and Stotzky, 1997]. The single stranded DNA molecules serves as templates for the synthesis of complementary strands in the donor and recipient. The recipients then give rise to transconjugants when transferred genetic material is stably inherited.

As a method of genetic transfer, conjugation is popular for genetically modifying non-bacterial cells, such as plant cells due to the minimal disruption of the recipients cellular envelope, compared to for example particle bombardment.

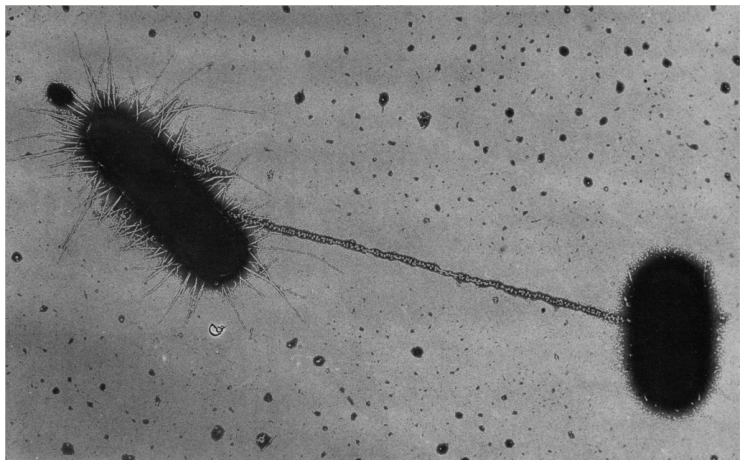


Figure 2.2: Scanning electron micrograph of *E. coli* bacteria taking part in conjugation. Between the two cells, the pilus used in bacterial conjugation appears almost like a tube. Pili and fimbriae are usually coated with viral proteins to be made visible in this manner [Madigan et al., 2015]. Image courtesy of Charles C. Brinton Jr. [www.nlm.nih.gov].

2.3.2 Donor strains

So far, we have discussed F+ cells as a donor strain being able to transfer DNA because they contain the F factor. There are in addition, strains that have chromosomally integrated F or functions for conjugal transfer and these

strains were termed Hfr strains for their high frequency of recombination by Cavalli-Sforza [Cavalli-Sforza and Jinks, 1956]. It was made evident that the Hfr strain possesed chromosomally integrated F factor when Hfr and F- crosses did not result in any F+ or Hfr cells. This is in contrast to F+ and F- crosses which typically result in a high percentage of conversions to F+ [Griffiths et al., 2000].

Today, such strains compromise an important asset in genetic engineering and biotechnology. Several types of bacteria have been studied and used as donor strains in conjugal transfer of DNA, such as many *Streptococcus* strains, as well as using virulent pathways of pathogens like *Legionella pneumophila* [Snook and McKay, 1981, Neve et al., 1984, Walsh and McKay, 1981, Vogel et al., 1998]. For obvious reasons *E. coli* remains the favored bacterium to work with in laboratories, and conjugation is not an exception. As mentioned earlier, the *E. coli* strain that several groups worked with in their early studies of conjugation was K-12. From their experiments they isolated Hfr strains and F+ strains. Scientists have been isolating and further selecting several *E. coli* strains possessing characteristics that make them desirable for diverse purposes in the laboratory. The most common *E. coli* strains used for conjugation are SM10 and S17-1. These strains are used to carry the broad-host-range plasmids of incP with RK2/RP4 *oriT*. SM10 and S17-1 have chromosomally integrated RP4 plasmid and therefore also enable the transfer genes for these plasmids. The donor strains also contain the genome of bacteriophage Mu, which can cause certain problems that will be discussed in Section 3.1.3 of Related work.

2.3.3 Transkingdom conjugation

Similar and almost identical to conjugation between bacteria, transkingdom conjugation occurs between species of different kingdoms.

The alphaproteobacterium *Agrobacterium tumefaciens* was linked to crown gall tumours in plants before the 1960s, and is perhaps the best known example of an organism that transmits its DNA into host cells of plants. Some years later, it was discovered that the causative agent of gall formation was DNA, called T-DNA, originally a part of the conjugative plasmid of *A. tumefaciens*, the Ti plasmid [Syvanen and Kado, 2001]. *Agrobacterium*'s tumor-inducing (Ti) and hairy root-inducing (Ri) plasmids are the main cause of crown-gall or hairy-root disease, respectively. They are also the basic tools for construction of transgenic plants [Suzuki et al., 2009]. Conjugation with *A. tumefaciens* is mediated by *vir*

genes.

In 1989 it was revealed that the same conjugative systems and plasmids that were mediating conjugation between bacteria could be used to mediate transkingdom conjugation in the yeast *Saccharomyces cerevisiae* [Heinemann and Sprague, 1989]. In that same study, Heinemann et al. described *E. coli*-yeast conjugation being dependent on the same *tra* genes that drive conjugation between *E. coli* with no additional plasmid-encoded requirements. The study also observed that DNA could be transmitted using a broad-host-range plasmid as well as narrow/limited-host-range plasmid. However, broad-host-range plasmids of the incP group are still preferred as they have been compared to plasmids of narrow-host-range plasmids from incF and incl1, showing that incP plasmids were able to transmit mobilizable plasmids into yeast under conditions where the incF and incl1 could not [Bates et al., 1998].

Unlike *A. tumefaciens* and plants, *E. coli* and yeast have no obvious ecological relationship and through similar experiments, it was discovered that transkingdom conjugation is not species-specific [Syvanen and Kado, 2001]. In additon to plants and yeast, successful conjugation has been reported in mammalian cells [Waters, 2001]. Since then, more organisms have been added to the list of recipients, such as diatoms, which are a closely related example to this project and will be described in the next chapter (Section 3.2).

It is also interesting to note that unicellular microalgae probably owe their existence to green bacteria that through endosymbiotic gene transfer drove the evolution of photosynthesis [Robertson and Tartar, 2006]. As mentioned before, the plastid of *Nannochlorpsis*, a heterokont, is derived from the secondary endosymbiosis. This is thought to have involved a heterotrophic host and an ancestor of the red algae, with the events occuring before the divergence of heterokonts and alveolates [Janouškovec et al., 2010].

3 Related work

This chapter describes previous related work, which serves as a basis for this project. Firstly, the plasmids used in this project, their construction and characteristics, will be detailed. A description of a study reporting conjugal transfer of episomes to heterokont algae, will follow.

3.1 Plasmids used in this study

3.1.1 pAPA1001 plasmids

Nguyen created in her thesis a set of novel plasmids with endogenous promoters for *N. oceanica* CCMP1779, called pAPA1001 [Nguyen, 2016]. The plasmids were made by insertion of promoter - terminator and reporter gene sequences into a pUC19 backbone. pUC19 (2,686 bp) is a high copy number *E. coli* plasmid containing an ampicillin resistance gene (*AmpR*); β-galactosidase gene (*lacZ*) under the control of lac promoter; a 54 bp multiple cloning site; and pMB1 origin of replication.

The promoter and terminator sequences were chosen from the genes *nanno-169* (from *Nannochloropsis* CCMP1779-3874) and *nanno-602* (from *Nannochloropsis* CCMP1779-11694) from the genome sequenced by RNAseq [Vieler et al., 2012]. Through microarray analysis, these genes showed high and stable expression rates under different conditions [Nguyen, 2016]. The promoter and termiantor fragments were synthesized by Eurofins Medigenomix. The *nanno-602* gene encodes a general substrate transporter with a major facilitator superfamily

domain and consists of a single exon. The gene *nanno-169* encodes a Clp protease also as a single exon. Both promoters are expressed twice as high as the LDSP promoter in pSELECT100 (section 3.1.2).

Six vectors were constructed by creating two vectors for each selectable marker gene, that have two different promoters, but the same terminator. Zeocin (*Sh Ble*), hygromycin B (*aph7*) and paromomycin (*aphH*) resistance genes were selected as marker genes. The expression cassettes were inserted into the pUC19 backbone by BamHI and SphI restriction and ligation. The constructs were named pAPA0602-X and pAPA0169-X (X-marker gene) after the promoter sequences they carried. The constructs were also designed to facilitate insertion of other genes for future work by accommodating BioBrick-compatible restriction sites (PacI and XhoI).

The plasmids used in this project, pAPA0602-Z (4116 bp) and pAPA0169-Z (4247 bp), carry the zeocin resistance casette, as those plasmids were the only set that resulted in successful transformation of *N. oceanica* [Nguyen, 2016]. Successful transformants were determined by colony PCR performed on 20 random colonies and transformation efficiency was reported to be $1.2\text{x}10^{-8}$ and $6.5\text{x}10^{-8}$ colonies/cell/µg DNA for pAPA0602 and pAPA0169, respectively. Both plasmids can be seen in Figure 3.1.

3.1.2 pSELECT100

pSELECT100 is a custom made plasmid by DNA cloning service (www.dna-cloning.com). It is made up of a 497pLC-SfiI plasmid, with LDSP promtoer region, *aph7* gene conferring resistance to Hygromycin B and a 35S terminator. The native LDSP promoter was amplified from *N. oceanica* CCMP1779 genome and blunt cloned to the dephosphorylalted 497pLC-Hpt-SfiI backbone to result in the selection plasmid pSELECT100 [Vieler et al., 2012]. The plasmid also includes an ampicillin resistance cassette and pBR2 origin. A plasmid map of pSELECT100 can be seen in Figure 3.2. The plasmid's LDSP promoter resulted in stable expression under nitrogen starvation.

Some disadvantages to pSELECT100 is that it contains several restriction sites in the promoter region and is not designed to facilitate homologous recombination [Nguyen, 2016]. Previous studies using this plasmid have reported low transformation efficiencies and a high level of false positives [Chernyavskaya, 2014].

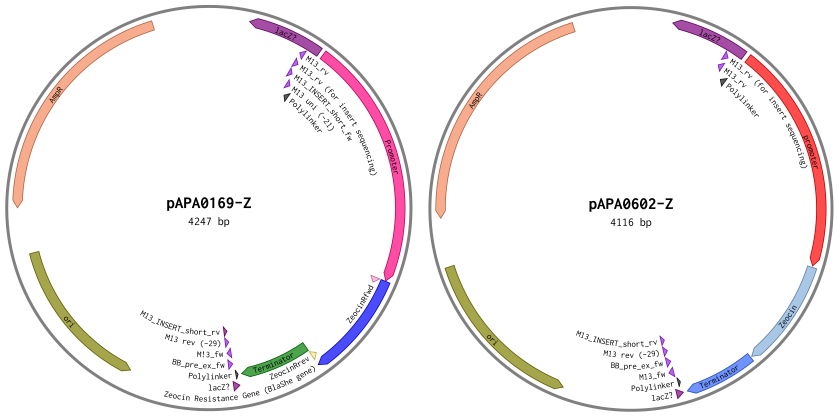


Figure 3.1: pAPA1001-Z plasmids carrying zeocin resistance cassette under endogenous promoters. Both plasmids are constructs of a pUC19 backbone and carry AmpR, ori and LacZ. (a) pAPA0169 is 4247 bp long and carries zeocin resistance gene under promoter nanno-169. (b) pAPA0602 is 4116 bp long and carries zeocin resistance gene under the nanno-602 promoter. Plasmid maps created in Benchling (www.benchling.com)

3.1.3 pTA-Mob

In an effort to create an improved and host-independent plasmid system of conjugal transfer, Strand et al. (2014) created the mobilization plasmid pTA-Mob [Strand et al., 2014]. In their study, drawbacks were detailed of the common *E. coli* strains, SM10 and S17-1, used in conjugation. The drawbacks include the mobilization of the Mu phage genome into recipient strains, random mutations of recipient genome or mobilized plasmid as a result of this, and the chromosomal DNA mobilization from the donor. This can present a problem due to the likeliness of gene inactivation in the mobilized plasmids [Strand et al., 2014].

pTA-Mob (Figure 3.3) is constructed as a broad-host-range (RK2-compatible) plasmid that includes all the functions required for conjugal transfer of *oriT*-containing vectors. The system is compatible with other replicons commonly used in conjugation experiments and can be used in diverse bacterial strains. The study reported the successful transfer by conjugation of large plasmids without

Figure 3.2: pSELECT100 is constructed from 497pLC-SfiI plasmid, carrying a native LDSP promoter amplified from *N. oceanica* CCMP1779 genome, aph7 gene conferring Hygromycin B resistance, 35S terminator, pBR2 origin and AmpR cassette.

the modifications observed under *E. coli* S17-1 conjugation.

3.2 Diatom transformation by conjugation

A study recently reported the first plasmid delivery method by bacterial conjugation to diatoms of the Stramenopile lineage, which are generally transformed by particle bombardment [Karas et al., 2015]. The study described the use of a nuclear episomal vectors and their delivery to the diatoms *P. tricornutum* and *T. pseudonana* by *E. coli*. The method was reported to be successful and the diatoms were able to maintain the delivered episomes with high fidelity for up to 30 generations.

The used protocols differ slightly between the two diatoms with the main differences being the cultivation media and incubation for different periods of time before selection. After incubating the recipient and donor cells for 90 minutes at 30 °C, *P. tricornutum* was moved into light and grown for 2 days at 18 °C before selection, while *T. pseudonana* was only grown for 4 hours before selection.

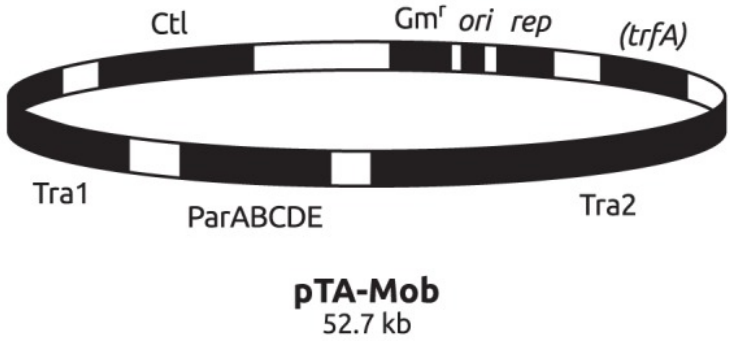


Figure 3.3: pTA-Mob (52.7 kb). Gm^r, gentamycin resistance gene; *rep*, pBBR1 replication gene; *ori*, pBBR1 replication origin; (*trfA*), replication initiation protein gene from RK2 replicon although inactive due to lack of *oriV*; Tra1 and Tra2, regions containing tra genes necessary for conjugative tranfer of *oriT* containing plasmids; parABCDE, stablization region encoding gene products ParA, -B, -C, -D, -E; Ctl, central control operon of RK2 [Strand et al., 2014].

3.2.1 Yeast *CEN6-ARSH4-HIS3* sequence

In the same study, Karas et al. (2015), identified a yeast-derived sequence that allowed stable episomal replication in the diatoms. Episomal plasmid replication was enabled even in absence of antibiotic selection with a copy number equal to that of native chromosomes. The sequence, *CEN6-ARSH4-HIS3*, is 1.4 kb long and allows the plasmids low-copy episomal replication in both diatoms. CEN6-ARSH4-HIS3, contains plasmid maintenance functions for yeast centromeric plasmids (*CEN6*, yeast centromere and *ARSH4*, autonomously replicating sequence), and *HIS3* gene that confers yeast histidine auxotrophy. How the sequence enables episomal replication, is not yet known. The sequence has an interesting GC content where *CEN6* is 13% GC and *ARSH4* has 30% GC. Low GC content is a characteristic of red algal and protist centromeres [Karas et al., 2015].

Conjugation from *E. coli* to *T. pseudonana* resulted in higher efficiency when the yeast sequence was included in the transferred plasmids. The *CEN6-ARSH4-HIS3* region can be amplified and added to any plasmid to allow episomal replication [Karas et al., 2015].

Part II

Materials and Methods

4 Growth media optimization

A series of growth experiments were conducted to determine which type of medium was the most suitable for the growth of *Nannochlorpsis*. Initially *Nannochloropsis* was cultured on the same media used for growing the algae throughout the whole project (Cell-Hi NC enriched artificial sea water). Results are reported in the Chapter 8.

4.1 Strain and growth conditions

N. oceanica strain CCMP1779 from the National Center for Marine Algae and Microbiota (www.ncma.bigelow.org) was cultured in enriched 50% artificial sea water (ASW). ASW is made with Instant Ocean® sea salt (www.instantocean.com), dissolved in distilled water to a concentration of 50% and autoclaved before supplementation. Cell-Hi NC medium from Varicon Aqua Solutions (www.variconaqua.com) is used for enrichment. To maintain a pure culture, ampicillin with a final concentration of 50 µg/mL was added to the culture, knowing that an ampicillin concentration of up to 300 µg/mL does not have detrimental effects on the algal cells [Chernyavskaya, 2014]. Algal cells were inoculated in 200 mL of enriched 50% ASW in a bubbling flask system at 25 °C under constant light.

Table 4.1: Composition of media used for growth experiment

Sea water	**Enrichment**	**Agar-agar content**
50% ASW	Cell-Hi NC medium	1.20%
50% FSW	F/2 medium	1.20%
50% FSW	Cell-Hi NC medium	1.20%
50% ASW	none	1.20%
50% FSW	none	1.20%

4.2 Media, washing and spotting

The different media types consisted of ASW and filtered sea water (FSW) enriched with Cell-Hi NC medium or Guillard's F/2 medium (Appendix A, Table A.1) [Guillard, 1975]. Two controls were established for both ASW and FSW that were not enriched. The media were prepared and autoclaved before some were enriched, as described in Table 4.1.

Algal cells were grown to a log phase (OD_{750} of 0.116) at a temperature of 25 °C in a bubbling flask system. The cells were collected by centrifugation at 5000 rpm for 5 minutes. The pellets were washed three times in nonenriched FSW. A dilution series was made to achieve samples with OD_{750} of 1, 0.1, 0.01 and 0.001. A volume of 5 µL droplets from each dilution were spotted in rows on the plates containing the different media (Figure 4.1). The droplets were allowed to dry under the sterile hood before the plates were sealed and incubated under constant light at 26 °C. Images were taken every 12 hours for 96 hours and then every 24 hours for 10 days. Imaging of the plates was carried out in an imaging setup that allowed measurements of relative cell densities for each droplet by use of a light-tight box with a fixed camera and constant light intensity from an LED source.

4.3 Image processing and analysis

After acquiring the images, an in-lab script written in Python 2.73 was used to measure density of growth. The RAW images were first converted to MRC format stacks and manually aligned using Midas software to make image stacks for each

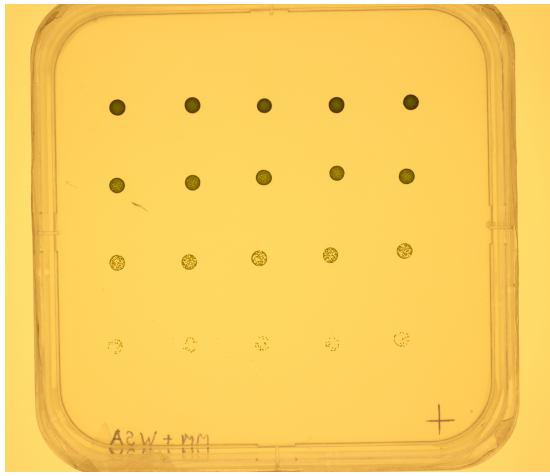


Figure 4.1: Example of plate used in growth experiment showing the various dilutions of the algal cells. From top: OD_{750} 1, 0.1, 0.01 and 0.001. The image was taken after incubation of 14 days on enriched ASW media. The cross on the bottom corner was made for alignment of images during processing.

plating media. The position of the colonies was then selected in each stack. Cell densities were then calculated by the script and compared to background in the plates surrounding the colonies. The data for each colony over times was compiled into a raw data file, which is was then analyzed by in a spreadsheet program (LibreOffice Calc).

5 Molecular cloning

A series of steps using molecular cloning techniques were carried out to generate the plasmids required for conjugation experiments. In this section, these steps will be described. Results can be viewed in Chapter 9.

5.1 Cloning of *oriT*

The pAPA1001 plasmids, pAPA0602 and pAPA0169, and pSELECT100 were all altered to render them suitable for conjugal transfer by inserting the origin of transfer (*oriT*).

5.1.1 Heat-shock competent cells

Heat shock competent cells are an important tool in molecular biology. In this project they play an intermediate role in carrying the constructs before they are isolated by plasmid extraction.

Cultures were prepared by inoculating 1 L of *E. coli* DH5α cells in 20 mL SOC (Appendix B, Table B.2a) and left on shaking at 225 rpm and 37 °C overnight. 10 mL of filter sterilized $MgSO_4$ was added to 300 mL yB media (Appendix B , Table B.2d) and warmed at 37 °C for 10 minutes. A volume of 3 mL of the overnight culture was inoculated in the yB media and left to grow in shaking incubator at 37 °C. Optical densities (OD) were measured to determine growth phase. After 2 hrs, and an OD_{600} of 0.438, the culture was divided into six pre-chilled falcon tubes and centrifuged at 4000 rpm for 10 minutes at 4 °C.

The supernatant was discarded as thoroughly as possible and the cell pellets resuspended in 15 mL of pre-chilled TfBI (Appendix B, Table B.2b). The cells were spun down immediately at 4000 rpm, 4 °C for 10 minutes. The supernatant was discarded and cell pellets were resuspended in 1 mL of pre-chilled TfBII (Appendix B, Table B.2c). Aliquots were made with volumes of 100 µL and snap frozen with liquid nitrogen. The tubes were immediately stored at –80 °C.

Heat shock transformation and transformation efficiency

The transformation efficiency was determined by heat shock transformation of the competent cells using pUC19. Heat shock competent DH5 cells were thawed on ice for 10 minutes. A volume of 1 µL of pUC19 was added and the mixture incubated for 30 minutes on ice. A negative control was also established by adding dH_2O to a second aliqot of cells to check for background resistance. The tubes were incubated for further 30 minutes on ice. Cells were then heat-shocked for 45 seconds in 42 °C and incubated for 3 minutes on ice. The cells were then transferred to tubes containing 2 mL of LB and incubated while shaking (225 rpm) for 90 minutes at 37 °C. Dilutions were made and 100 µL of each cell culture was plated out on LB-Agar plates containing 100 µg/mL ampicillin. Plates were incubated overnight at 37 °C and colonies were counted. Transformation efficiency is calculated as the number of colony forming units (cfu) per nanograms of DNA. An example is shown in Appendix B and raw data presented in Table B.1.

Primer design and amplification of *oriT*

Firstly, single cutters for both pAPA0001 and pSELECT100 plasmids were identified: SapI for pAPA1001 plasmids and KpnI for pSELECT100. Primers for *oriT* amplification were designed on Benchling (www.benchling.com) to contain SapI and KpnI restriction sites (highlighted) in the overhangs as shown in Table 5.1.

Using the primer pairs (Table 5.1, a sequence containing *oriT* was amplified from pHH100 plasmid with an expected length of 487 bp long for pAPA1001 and of 481 bp for pSELECT100 (Appendix B, Figure B.1). Q5® High-Fidelity DNA Polymerase was used and the reaction and program are described in Tables

5.2 and 5.3 Primer melting temperatures were determined using New England BioLabs® T_m calculator (www.tmcalculator.neb.com).

Table 5.1: Primers desgined for amplification of oriT-containing sequence within pHH100 plasmid. Restriction sites for SapI in pAPA1001 primers and KpnI in pSELECT100 primers are highlighted.

pAPA1001	
forward	5'-TAAGGCTCTTCNGCTAGACACAACGTGGCTTTCCC-3'
reverse	5'-ATCTGCTCTTCNAGCGAAAGGGGGATGTGCTGCAA-3'
pSELECT100	
forward	5'-TAAGCAGGTACCAGACACAACGTGGCTTTCCC-3'
reverse	5'-TGCTTAGGTACCGAAAGGGGGATGTGCTGCAA-3'

Table 5.2: Q5® High-Fidelity DNA PCR reaction components.

Component	**50 µl Reaction**	**Final concentration**
5X Q5 Reaction Buffer	10 µl	1X
10 mM dNTPs	1 µl	200 µM
10 µM OriT-F	2.5 µl	0.5 µM
10 µM OriT-R	2.5 µl	0.5 µM
pHH100	1 µl	<1,000 ng
Q5 High-Fidelity DNA Polymerase	0.5 µl	0.02 U/µl
Nuclease-Free Water	to 50 µl (32.5 µl)	

The amplified sequences were confirmed to be the correct size by gel electrophoresis (Appendix B, Figure B.3). The PCR products were then purified using Qiagen's Qiaquick® PCR purification kit.

5.1.2 Digestion-ligation cloning and transformation

Digestion reactions were set up for pSELECT100, pAPA1001 plasmids and PCR products in 10 µL according to protocol of New England BioLabs®. The reactions were incubated for 1 hour at 37 °C. Reactions containing pAPA1001 and pSELECT100 were treated with calf-intestinal alkaline phosphatase (CIP), by adding 1 µL of CIP to the reactions and incubating further for 1 hour.

Table 5.3: Q5® High-Fidelity DNA PCR program.

Number of cycles	**Temperature** (°C)	**Duration**	**Step**
1	98	30 sec	Initial denaturation
	98	10sec	Denaturation
30	65	20sec	Annealing
	72	20 sec	Extension
1	72	2 min	Final Extension
1	4	∞	Hold

Correct sizes of the linearized plasmids were confirmed via gel electrophoresis. All digestions were purified using the (Qiagen's Qiaquick®) PCR purification kit and DNA concentrations were measured using NanoDrop^{TM}2000 spectrophotometer (Thermo Fisher Scientific).

Ligation reactions were set up according to New England BioLabs® Quick Ligation^{TM} protocol. A 1:3 vector-insert ratio was applied. Ligations were incubated at room temperature for 5 minutes used directly for heat shock transformation of competent DH5α cells. Transformation cells were plated on LB-Agar plates containing 100 μg/mL ampicillin overnight at 37 °C.

The following day, colony PCR was performed to screen for transformants carrying the insert. For this, colonies were picked with a sterile pipette tip and resuspended in 30 μL of dH_2O and 1 μL was used as template for PCR, as described in Tables 5.2 and 5.3, but with a longer (6 minutes) initial denaturation step. Presence of insert was confirmed by gel electrophoresis.The constructs were then extracted by plasmid isolation using Qiagen's QiaPrep® Spin Miniprep kit.

Confirmation by sequencing

Single sequencing primers were made for confirmation of inserted *oriT* sequence, shown in table 5.4. The sequencing samples were prepared in 10 μL volumes containing around 500 ng of DNA, 5 μL of 5 μM sequencing primer and sterile dH_2O if necessary. The samples were sequenced by GATC Biotech and the results were analyzed and aligned on Benchling (chapter 9).

Table 5.4: Sequencing primers for *oriT*-containing sequence in pAPA1001 and pSELECT100 plasmids.

Seq.pAPA-oriT	5'-TGTAAAGCCTGGGGTGCCTAATG-3'
Seq.pSEL-oriT	5'-AACCCTGCTTCGGGGTCATTATAGC-3'

5.2 Cloning of *CEN6-ARSH4-HIS3* sequence

In addition to *oriT*, the pAPA1001 plasmids were also chosen to additionally include, the yeast sequence, C*EN6-ARSH4-HIS3*, known to allow plasmids to exist as low copy episomes in the diatoms *P. tricornutum* and *T. pseudonana* [Karas et al., 2015].

5.2.1 Gibson assembly

The *CEN6-ARSH4-HIS*3 sequence was cloned via Gibson assembly, an assembly method developed by Daniel G. Gibson that allows assembly of several DNA fragments in a single reaction taking 15-60 minutes [Gibson et al., 2009]. The assembly reaction is driven by the actions of three crucial enzymes; an exonuclease, DNA polymerase and DNA ligase. The exonuclease chews back the 5'-ends to create single-stranded 3'-ends to enable annealing of complementary overhangs of the assembly fragments. DNA polymerase then extends the 3'-ends, filling in the gaps created by the exonuclease after annealing, and DNA ligase seals nicks, leaving seamlessly assembled construct.

The assembly fragments for Gibson cloning were created by PCR amplification. Primers containing overhangs that were sufficiently long to successfully overlap between the fragments were designed using online tools on Benchling. The *CEN6-ARSH4-HIS3* (insert) sequence was amplified from ptpPUC3. Refer to Appendix B, Figure B.2 for sequence and Table B.3 for primers. The PCR reaction was performed using CloneAmp^{TM} HiFi PCR (Takara), which allows amplification of large fragments in about one hour due to its three-step program (Tables 5.5 and 5.6). Correct sizes of amplified fragments were checked via gel electrophoresis. The fragments were then treated with DpnI to digest methylated DNA, leaving only the PCR products by adding 19 µL dH_2O, 5 µL CutSmart buffer (NEB)

and 1 µL DpnI, making a total volume of 50 µL. The reaction was run for 1 hour at 37 °C. The fragments were then purified using Qiagen's PCR purification kit. Concentrations were measured with NanoDrop™2000 to prepare the Gibson Assembly® reaction according to Table 5.7. The reactions were incubated at 50 °C for 1 hour. A volume of 5 µL of the Gibson assembly reactions were used to transform DH5α *E. coli* cells with heat shock transformation for 45 seconds at 42 °C. Cells were incubated for 90 minutes at 37 °C in 1 mL of LB before spinning down, resuspending in 100 µL LB and plating out on 100 µg/mL ampicillin LB-Agar. Plates were incubated at 37 °C overnight.

Table 5.5: CloneAmp™ HiFi PCR reaction components.

Reagent	**25 ml reaction**
CloneAmp PCR pre-mix	12.5 µl
10 µM FWD primer	0.5 µl
10 µM REV primer	0.5 µl
template	<100 ng
Nuclease-Free Water	to 25 µl

Table 5.6: CloneAmp™ HiFi PCR program.

Number of cycles	**Temperature** (°C)	**Duration**	**Step**
	98	10 sec	Denaturation
35	55	7 sec	Annealing
	7 2	5 sec/Kb	Extension
1	4	∞	Hold

Table 5.7: Gibson Assembly® reaction for pAPA1001 plasmids and *CEN6-ARSH4-HIS3* sequence.

Reaction	**Backbone** (ng)	**Insert** (ng)	**Gibson Assembly master mix** (µL)
pAPA0602	<100	100	15
pAPA0169	<100	100	15

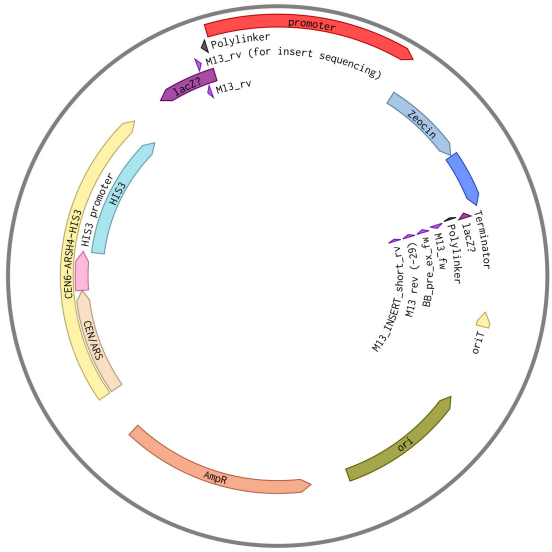


Figure 5.1: pAPA1001-YST, the result of Gibson assembly of pAPA1001 backbone and insert fragment *CEN6-ARSH4-HIS3* as visualized by Benchling. The construct pAPA1001-YST also carries an ampicillin selection marker for *E. coli*, *oriT* and a zeocin resistance gene under an endogenous promoter in *N. oceanica*.

Confirmation of successful assembly

The assembled constructs were checked in three different ways for insertion of the desired sequence. Firstly, a colony PCR and gel electrophoresis were performed to confirm the presence of the inserted *CEN6-ARSH4-HIS3* sequence. Colonies that were shown to contain the constructs were used for plasmid isolation. Secondly, insertion and correct size of the construct was confirmed by restriction digestion. Finally, sequence confirmation was achieved by sequencing. Primers for sequencing were the same used to amplify the sequence of interest *CEN6-ARSH4-HIS3* (Appendix B, Table B.3). The yeast sequence is 1.4 kb, which is too long to sequence in one read. Additionally, the sequence reads can become inaccurate at both ends. Therefore, several samples of the plasmids were sent to ensure that the whole region was sequenced. Sequencing samples were prepared as described previously and the samples were sequenced by GATC Biotech . The results were analyzed and aligned on Benchling. Constructs of the vectors pAPA0602 and pAPA0169 that include the yeast sequence will be termed pAPA0602-YST and pAPA0169-YST, respectively. Refer to Chapter 9 for sequencing results.

6 Establishing conjugation protocol for *N. oceanica*

This chapter describes methods and experiments that were applied and resulted in successful conjugation. Furthermore, the methods that were unsuccessful in generating transconjugants will also be detailed. This is a reverse chronological order of how the protocol was developed in reality, as many unsuccessful attempts were made in the beginning, before successful conjugation was established. Results can be viewed in Chapter 10.

Assessment of co-incubation media

Due to different nutritional requirements of *Nannochloropsis* and *E. coli*, three solid media types for co-incubation were made. The first was made of one half *Nannochlorpsis* medium (50% ASW enriched with Cell-Hi NC medium) and one half LB. The second was *Nannochloropsis* medium with 1% peptone added and the third, purely *Nannochloropsis* medium.

The media types were tested by using them in a trial conjugation between *Nannochloropsis* and *E. coli* strain S17-1 carrying pAPA0602 and pAPA0169. After incubation of the conjugation mix on the differing plating media, growth was assessed, and based on the results, one plating media was chosen for all subsequent co-incubations.

Selective media

Media for selection of transconjugants are generally made suitable for the growth of the recipient cells while containing a chemical that eliminates growth of cells that did not receive the selection marker, such as an antibiotic. This eliminates also the donor cells, leaving only the transconjugants able to grow. The pAPA1001 plasmids confer zeocin resistance while pSELECT100 confers hygromycin resistance, with the lethal doses for wild type *N. oceanica* being around 2 µg/mL and 50 µg/mL, respectively. Since the lethal concentrations for *E. coli* are up to 200 µg/mL for hygromycin and 25-50 µg/mL for zeocin, an additional antibiotic with natural selective effects on *E. coli*, but not the algae can be used against the growth of *E. coli*. Kanamycin at concentrations up to 300 µg/mL has no detrimental effect on the growth of *N. oceanica* [Chernyavskaya, 2014], and it was used in selective plates at concentration of 20 µg/mL to eliminate *E. coli* growth. Both types of selective media were controlled by plating wild type *N. oceanica*.

6.1 Donor strain *E. coli* S17-1

After confirming the successful insertion of *oriT* and *CEN6-ARSH4-HIS3* by sequencing, the plasmids were introduced to the donor strain *E. coli* S17-1. Five different plasmids were introduced to *E. coli* strain S17-1 by heat shock transformation; the pAPA1001 plasmids including *oriT*, the pAPA1001 plasmids including *CEN6-ARSH4-HIS3* and *oriT*, and pSELECT100 including *oriT*.

Once donor strains were established, glycerol stocks were made by mixing 500 µL of 50% sterile glycerol with 500 µL of the cells, making a final concentration of 25% glycerol. Stocks were stored at –80 °C.

6.1.1 Testing functionality of *oriT* by bacterial conjugation

Bacterial conjugation was carried out between DH5α cells containing pHH100 (recipient) and S17-1 containing pAPA1001 plasmids (donor) to establish that the cloned *oriT* in pAPA1001 plasmids is functional. Plasmid pHH100 carries a kanamycin selectable marker, while the pAPA1001 plasmids carry an ampicillin

marker. If conjugation occurs and is successful indicating the presence of a functional *oriT*, the recipient cells should carry both pHH100 and pAPA1001 plasmids, and the cells are expected to have both kanamycin and ampicillin resistance.

Donor strains S17-1 carrying pAPA0602, S17-1 carrying pAPA0169 along with recipient strain DH5α carrying pHH100 were cultured overnight with selection. Dilutions of 1% cultures of donor and recipient cultures were made by inoculating 1 mL of the overnight cultures in 100 mL of LB without selection. The cultures were incubated on shaking (225 rpm) for 2 hours at 37 °C. A volume of 2 mL of the donor strain cultures were mixed with 2 mL of recipient cells and together centrifuged at 4000 xg for 5 minutes. The supernatant was removed, leaving 100 µL for resuspension. From each conjugation mix, 100 µL were spotted on a non-selective LB-Agar plate and left to incubate at 37 °C for 30 minutes. The growth from each spot was scraped up with an inoculation loop and resuspended in LB before plating out on selective LB-Agar containing 50 µg/mL ampicillin and 25 µg/mL kanamycin. The plates were left to incubate overnight at 37 °C.

6.1.2 Conjugation protocol using *E. coli* S17-1

This protocol is inspired by and based on the conjugation protocol for *P. tricornutum* and *T. pseudonana* [Karas et al., 2015]. After transformation of S17-1 strain of *E. coli*, conjugation can be performed as long as a log phase culture of the algae is available. Since *N. oceanica* can take up to a week to reach exponential growth (10^6 cells/mL), it was necessary to maintain cultures constantly at exponential phase by regularly replacing fresh medium to the cultures, as well as keeping several at different growth phases. The OD_{750} was measured for the algal cultures to determine growth phase and an OD_{750} between 0.9 to 0.13 indicated the presence of sufficient cells in log phase growth.

Overnight cultures of S17-1 cells carrying the various plasmids are prepared with antibiotic (100 µg/mL ampicillin) containing medium and are used as inoculum the following day for 1% cultures without antibiotics. The cultures were incubated at 37 °C with shaking at 225 rpm. Cultures were incubated for 2.5-3 hours or until OD_{600} was 0.4-0.6. *E. coli* cultures were centrifuged at 2000 xg for 8 minutes and resuspended in 1-3 mL of LB, depending on what ratio is desired between recipient and donor. Using Thermo Fisher's Sorvall LYNX 400 centrifuge, 200 mL cultures of *N. oceanica* CCMP1779 were centrifuged at 10 000

rpm (15 680 xg) for 10 minutes and then resuspended in 500 µL of enriched 50% ASW. Equal volumes of donor and recipient cells were mixed to ensure equal conditions for both donor and recipient cells. The total volume of the conjugation mixtures were between 400-1000 µL. Co-incubation plates consisting of enriched 50% ASW, 1% peptone and 1.2% agar-agar were prepared by placing FTA® Whatman filter membranes (Merck) using sterile tweezers on the agar plates. Conjugation mixtures in 50-80 µL volumes of various dilutions were spotted on membrane papers and left under the sterile bench for 10 minutes, so the liquid could be absorbed by the plates. The plates were then incubated in the dark for 90 minutes at 30 °C, before overnight incubation at 23 °C under constant light intensity of 76.5 µPAR. The membranes containing the conjugation mixtures were then moved, using sterile tweezers, onto selective plates containing 2 µg/mL zeocin and 25 µg/mL kanamycin for pAPA1001 transformations, or 50 µg/mL hygromycin and 25 µg/mL kanamycin for pSELECT100 transformations.

An image of a selective plate with membrane filters can be seen in the Figure 10.2, Chapter 10.

6.2 Donor strain *E. coli* DH10B carrying pTA-Mob

All plasmids were also introduced to the donor strain *E. coli* DH10B harbouring the conjugational plasmid pTA-Mob.

6.2.1 Compatibility between pBBR1 and, pBR2 and pUC origins

Plasmids with the same origins (ORIs) are usually incompatible because they compete for the same machinery and this can create an unstablity in retaining both plasmids [Novick, 1987]. Therefore, plasmids with the same ORI should not be co-transformed and that is why compatibility needs to be tested beforehand.

The compatibility of the pAPA1001 (pUC origin), pSELECT100 (pBR2 origin) and pTA-Mob (pBBR1 origin) is tested by making recipient DH5α cells containing pHH100 (pBBR1 origin) electrocompetent and transforming them with pAPA0602, pAPA0169 and pSELECT100 by electroporation.

Diluted 1% cultures were made of recipient cells by inoculating 1 mL of an overnight culture in 100 mL of LB with selection (50 µg/mL kanamycin). The culture was placed in shaking incubator at 37 °C for 2 hrs and 45 minutes. In pre-chilled microcentrifuge tubes, 1 mL of the culture was pelleted at 12 000 xg and 4 °C for 1 minute. The cells were washed three times in 1 mL of ice cold 10% glycerol. For final resuspension, 950 µL of the glycerol was removed and the cell pellet resuspended in the remaining 50-60 µL. Between 180-250 ng of the plasmids were added to the cells. The cell/DNA mixtures were transferred to chilled 2 mm electroporation cuvettes. Electroporation was conducted by setting Bio-Rad's GenePulser to exponential decay at 2500 V with 200 Ω resistance and 25 µF capacitance. Cells were immediately transferred to tubes containing 1 mL of LB and incubated with shaking (225 rpm) at 37 °C for 1 hour. A volume of 100 µL of cells were plated out on selective LB-Agar containing 25 µg/mL kanamycin and 50 µg/mL ampicillin and incubated overnight at 37 °C.

6.2.2 Creating two-plasmid systems for conjugal transfer

As previously mentioned in 2.3, conjugal transfer can occur either in strains that have chromosomally integrated transfer functions, or strains that harbor mobilization plasmids that carry the transfer functions. The latter can be called a two-plasmid system and the donor DH10B/pTA-Mob is an example of such a system.

An overnight culture was made by inoculating cells from glycerol stock of DH10B/pTA-Mob cells in 5 mL LB with 20 µg/mL gentamicin. The culture was incubated overnight at 37 °C with shaking (225 rpm). The following morning, a 1% culture was made with gentamicin selection and left on shaking for 3 hours at 37 °C. A volume of 1 mL of the culture was pelleted in pre-chillled microcentrifuge tubes for 1 minute at 12 000 xg rpm and 4 °C . The cell pellet was washed 3 times in chilled 10% glycerol and pelleted. Final resuspension volume was 50 µL. 150-200 ng of plasmid DNA was added to each aliquot of electrocompetent cells; pAPA0602, pAPA0169, pAPA0602-YST, pAPA0169-YST and pSELECT100. The cell/DNA mixtures were transferred to pre-chilled 2 mm electroporation cuvettes and the cells were electroporated at 2500 V with 200 Ω and 25 µF. The cells were then immediately transferred to 1 mL of LB in a microcentrifuge tube and placed on shaking at 37 °C for 1 hour. 50 µL of each donor cell was plated out on 15 µg/mL gentamicin and 50 µg/mL ampicillin LB-agar and incubated overnight at37 °C.

Glycerol stocks were made for established donor strains by mixing 500 µL 50% sterile glycerol with 500 µL of the cells (final concentration 25%) for storing at –80 °C.

6.2.3 Conjugation with DH10B/pTA-Mob

Conjugation by using donor strain *E. coli* DH10B/pTA-Mob is the same as described in Section 6.1.2, with the exception of the overnight cultures of the donor cells containing instead the antibiotics gentamicin at 15 µg/mL and ampicillin at 50 µg/mL.

6.3 Optimization of conjugation protocol

Except for the varying parameters of which donor strain is being used, three other parameters were tested, as described in the following subsections. For the following experiments, the pTA-Mob-carrying DH10B *E. coli* was chosen as donor strain, because as previously described it was expected to give more reliable results than S17-1 strain which could potentially cause mutations [Strand et al., 2014]. The experiments were also conducted using only one plasmid; pAPA0169.

6.3.1 Donor to recipient ratio

The ratio of recipient to donor in previous experiments in this project were carried out using ratios varying between 1:2 to 1:7. Studying the effect of the recipient to donor ratio on the conjugation efficiency is important. Previous studies have reported that tipping the ratio in favor of the donor, tends to give higher efficiencies [Dominguez and O'Sullivan, 2013].

Ratios were estimated by first finding the bacterial and algal cell number per µL of the conjugation mixture. The OD_{600} values of the bacterial cultures were always recorded before each experiment and then later used to find the cells/mL by using Agilent's '*E. coli* Cell Culture Concentration from OD_{600} Calculator'. The number of cells/mL for *N. oceanica* was found by correlation on a predetermined regression based on cell numbers determined by Flow Cytometer and OD_{750} values [Chernyavskaya, 2014].

An OD_{600} and volumes for resuspension and conjugation mixtures were decided on beforehand to approximate cell numbers to what is needed to achieve the exact ratios of 1:4, 1:2, 1:1, 2:1 and 4:1. Cell numbers, volumes and exact ratios are shown in Table 6.1. Conjugation between DH10B donor carrying pTA-Mob and pAPA0169 and *N. oceanica* was set up according to protocol described in Section 6.1.2.

Table 6.1: Recipient and donor cell numbers and volumes used for the ratio experiment.

Desired ratio	Donor volume	Donor cell number	Recipient volume	Recipient cell number	Actual ratio
1:4	125 µL	1.55 x 10^9	125 µL	3.3 x 10^8	1:4.7
1:2	100 µL	6.2 x 10^8	100 µL	2.64 x 10^8	1:2.4
1:1	200 µL	6.2 x 10^8	250 µL	6.6 x 10^8	1:1.1
2:1	200 mL	3.1 x 10^8	250 µL	6.6 x 10^8	2.1:1
4:1	200 mL	1.55 x 10^8	250 µL	6.6 x 10^8	4.3:1

6.3.2 Duration of co-incubation at 30 °C

Various co-incubation times at 30 °C were tested, in order to determine the influence of co-incubation time on the conjugation efficiency and to understand how rapidly the the process can occur. Incubations were performed at 10, 30, 90 and 180 minutes. The experiment followed the same protocol described in Section 6.1.2.

6.3.3 Co-incubation under light at 30 °C

In addition to co-incubation in the dark (6.3.2), the same times (10, 30, 90, 180 minutes) were tested for conjugation mixtures under a constant light of 71.5 µPAR. Protocol parameters in Section 6.1.2 were followed.

6.3.4 Conjugation efficiency

The efficiency of conjugation was calculated as the ratio between the number of transconjugants and the number of recipient cells that were plated. The spots that were made on the membrane filter paper when plating conjugation mixtures were of equal volume. Thus, they serve as replicates which enable the calculation of average efficiencies and standard deviations. Refer to Section 12.3.4 for discussion on the replicates.

6.4 Preliminary work

Prior to establishing the "standard" protocol (Section 6.1.2), many experiments were set up in an effort to establish conjugation parameters. The work was built on the original bacterial conjugation protocol described in Section 2.3 and the protocol used in the study by Karas et al. (2015). The preliminary work is distinguished from the protocol that was later developed (Section 6.1.2) by lacking the step of co-cultivation at 30 °C for 90 minutes before co-cultivation overnight at conditions suitable for algal growth. All the experiments were performed using both S17-1 and DH10B/pTA-Mob donor strains as well as both pAPA0169 and pAPA0602 plasmids, adding the listed variations as the work progressed:

- Replacing the bacterial recipient cells with cells of *N. oceanica*.
- Increasing cell numbers of both recipient and donor by 100-fold.
- Co-cultivation for 2 hours vs. overnight vs. 36 hours.
- Co-cultivation temperatures between 24 and 27 °C
- Varying recipient-donor ratios between 1:1.5, 1:2, 1:20, 1:50 and 1:100.

7 Electroporation

Electroporation was performed as a control and comparison for DNA transfer by conjugation as well as for the evaluation of the plasmids pAPA0602 and pAPA0169. Results are reported in Chapter 11.

7.1 Electroporation of *N. oceanica*

Electroporation has been described in detail in Section 2.2.

Plasmids used for electroporation were prepared by linearizing large quantities of plasmid DNA. To achieve a minimum of 2 µg DNA, digestion reactions were prepared according to New England BioLabs® CutSmart protocol, but in total volumes of 100 µL. The amount of plasmid DNA used in the digestion reactions often exceeded 3 µg to account for losses during purification. The reactions were run for 2 hours and 1 µL was using to confirm linearization by gel electrophoresis. Purification of the reactions were performed using Qiagen's Qiaquick® PCR purification kit. The smallest possible amount (0.5 µL) was used to measure concentration of the DNA after purification to ensure enough DNA is present (Table 7.1). The purified plasmid DNA was then concentrated by evaporation when the DNA was incubated in a microcentrifuge tube at 60 °C with the tube lid off. The tube was vortexed and spun down before and during incubation, until the volume remaining reached 5-10 µL. The DNA was either stored at –20 °C or used in electroporation. The restriction enzymes used were HindIII, EcoRV, EcoRI and BamHI (New England BioLabs®).

The electroporation protocol used is based on the previously reported work of

[Kilian et al., 2011] and [Vieler et al., 2012]. Similarly to conjguation, *N. oceanica* cultures need to be in exponential phase for transformations and maintenance of suitable cultures is necessary. Algal cells from 200 mL cultures (OD_{750} between 0.09-0.1) were collected using Thermo Fisher's LYNX Sorvall 400 F-14 centrifuge at 10 000 rpm (15 680 xg) and 4 °C for 12 minutes. The cell pellets were then resuspended in 25 mL of ice cold 375 mM sorbitol and transferred to 50 mL centrifuge tubes before centrifugation at 4 °C and 6000 rpm (5635 xg) for 5 minutes. This step was repeated twice more before the cells were resuspended in 1 mL in a microcentrifuge tube and centrifuged again before final resuspension in 200 µL of sorbitol. Linearized plasmid DNA with volume 5-10 % that of the cells was added to each aliquot of cells and mixed before being transferred to pre-chilled 2 mm WVR electroporation cuvettes. All steps were preformed on ice. Electroporation was conducted using BioRad's GenePulser, set to exponential decay at field strength of 2200 V, resistance of 600 Ω and capacitance of 50 µF. The amount of cells and DNA used for each transformation is detailed in Table 7.1. After pulsing, the cells were immediately resuspended in 10 mL of enriched 50% ASW without antibiotics and left to recover overnight at 23 °C, 100 rpm shaking and 76.5 µPAR light. The cells were then collected in centrifuge tubes and spun down at 5000 rpm for 5 minutes and plated out at various dilutions on selective media containing enriched 50% ASW 1.2% agar containing 2 µg/mL zeocin for pAPA1001 plasmids and 50 µg/mL hygromycin for pSELECT100. The plates were then incubated at 23 °C under constant light of 76.5 µPAR.

Transformation efficiency was calculated as described in Equation 7.1 below.

$$\text{Transformation effciency} = \frac{(\text{cfu} \div \text{plated cells})}{\mu\text{g DNA}} \tag{7.1}$$

7.2 Evaluation of pAPA1001 promoters

The novel plasmids pAPA1001 carrying the zeocin resistance casette under the promoters of *nanno-169* and *nanno-602* had not been previously evaluated. Therefore, the tolerance to zeocin conferred by the promoters was assessed.

Electroporation was performed according to protocol described in Section 7.1 for the plasmids pAPA0602 and pAPA0169, using the same cell number and amount of DNA described in Table 7.1. After allowing the transformed cells to

Table 7.1: Amount of plasmid DNA and cells used in electroporation experiment.

Plasmid	**Total cells**	**Plasmid DNA (μg)**
pAPA0169	$9x10^8$	2.875
pAPA0602	$9x10^8$	3.155
pAPA0169-YST	$9x10^8$	2.24
pAPA0602-YST	$9x10^8$	2.71
pSELECT100	$9x10^8$	2.058

recover overnight, they were collected and plated at on selective media containing varying antibiotic concentration: 2 μg/mL, 4 μg/mL, 8 μg/mL and 16 μg/mL zeocin. The plates were then incubated at 23 °C under constant light intesnity of 76.5 μPAR.

Part III

Results

8 Growth media optimization

In nature *Nannochloropsis* grows in natural ocean water, however in laboratories the standard *Nannochloropsis* growth medium is Guillards F/2 medium, which contains filtered sea water and supplement solution [Guillard, 1975]. Growth is an important factor for developing mutant strains. Therefore, an improved growth medium that provides faster and enhanced growth was developed and compared with established growth media.

Samples of *N. oceanica* culture (OD_{750} 0.1) were plated in 5 µ spots on the following media for evaluation: artificial sea water (ASW) enriched with Cell-Hi NC medium, filtered sea water (FSW) enriched with Cell-Hi NC medium, FSW enriched with F/2 medium, non-enriched FSW and non-enriched ASW. The growth was followed by imaging plates and growth determined using a Python script (Section 4).

Two days after initiation of the growth experiment, there was a significant difference between the growth on plates that were enriched with either Cell-Hi NC or F/2 medium and the ones that were non-enriched. Growth on the three types of enriched media grew more cells over time and seemed to maintain a "healthy" green color. There was no observable difference between those three types of media and it remained so during the whole experiment. The growth on non-enriched media (ASW and FSW) however, never developed a green color. Approximately a week into the experiment, growth on non-enriched media were observed growing in the same manner as the growth on enriched media, but possessed no color. The plated spots or "colonies" had the same densities, which varied according to dilution when plating, as other green colonies. This did not change until termination of the experiment.

After acquiring all the images and processing them according to procedure described in Chapter 4, growth data was analyzed to create growth curves based on the colony densities. The averages and standard deviations were calculated based on five replicates for each colony. The density is calculated by the script as a relative factor and therefore has no unit.

For the reasons stated above, images of the plates containing non-enriched media were not included in the analysis. Densities and growth could not be determined in cells without color. The growth curves presented belong to samples plated at an $OD_{750} = 0.1$, because at this concentration the initial growth could be resolved well. Figure 8.1 shows a comparison between the growth of colonies of the same dilution on the three media. It appears that colonies growing on ASW enriched with Cell-Hi NC have a higher average density than the other media. Growth on FSW + Cell-Hi NC comes second and FSW + F/2 last.

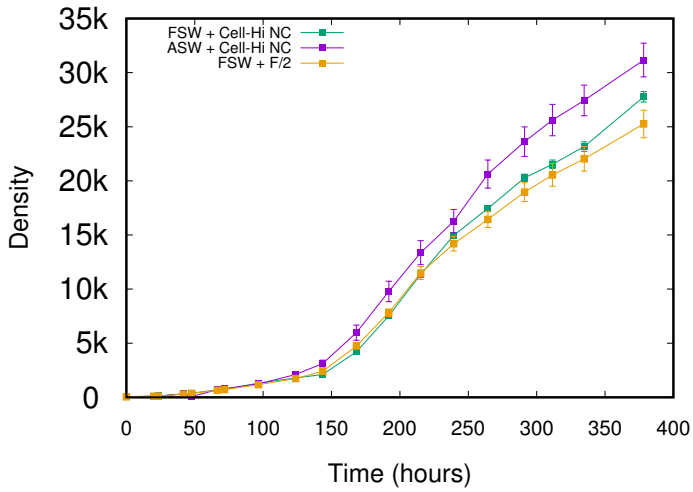


Figure 8.1: Comparison of three media based on growth curves generated by measurement of colony density of *N. oceanica*. Artificial sea water (ASW) enriched with Cell-Hi NC medium (purple), filtered sea water (FSW) enriched with Cell-Hi NC medium (green), FSW enriched with F/2 medium (orange). The measurement spanned 14 days with a starting OD_{750} of 0.1. Raw data was generated using a Python script and later analyzed in LibraOffice Calc. The data points and error bars represent the average density and standard deviation calculated from five replicates.

9 Molecular cloning

The origin of transfer (*oriT*) needs to be included for the transfer of plasmids via conjugation. As previously discussed in section 2.3, the yeast sequence *CEN6-ARSH4-HIS3* has been found to allow plasmids to exist as episome in the diatoms *P. tricornutum* and *T. pseudonana*. *CEN6-ARSH4-HIS3* was included in the project hoping that it could improve the efficiency and probability of incorporation of DNA constructs into the genome of the host organism.

Presented here are the results of the molecular cloning of the three plasmids pSELECT100, pAPA0602 and pAPA0169. *oriT* was cloned into the mentioned plasmids, while the yeast sequence *CEN6-ARSH4-HIS3* was cloned into pAPA0602 and pAPA0169, creating pAPA0602-YST and pAPA0169-YST. Digestion-ligation cloning and Gibson assembly techniques were applied to create the five constructs.

Efficiency of competent cells

After making heat shock competent DH5α cells and transforming a few aliquots with pUC19, the transformation efficiency was determined to be between 1.0 x10^7 and 1.90 x10^6 (refer to Appendix B, Table B.1 for data and calculation).

9.1 Sequencing results

Insertion of correct *oriT* and *CEN6-ARSH4-HIS3* region was confirmed by sequencing and the results analyzed and aligned in the online tools of Benchling. Figures 9.1, 9.2 and 9.3 show sequencing results of the regions within the plasmids

containing the cloned *oriT*. All results show a complete match when aligned against the *oriT* sequence online. In the chromatogram is more baseline noise for pAPA0602-oriT (Figure 9.1) when compared to the two other results.

Figures 9.4, 9.5 and 9.6 show the regions within the pAPA1001-YST plasmids that have the inserted *CEN6-ARSH4-HIS3* sequence. The noise and inaccurate base calling is expected at the beginning and end, and can be seen in all the alignments in the form of red peaks that are inverse to the chromatogram peaks. Because the sequencing result and the template is relatively large and the whole region is included in the Figure, the peaks and base calls do not appear clearly.

Looking closely at Figures 9.4 and 9.5, some mismatch is clear in the region of the HIS3 gene, around 4200 bp on the plasmid axis. The mismatch appears to be a conversion from CT to GG followed by an insertion of CGT. The mismatches are only three bases apart and the can be seen clearly in Figure 9.7.

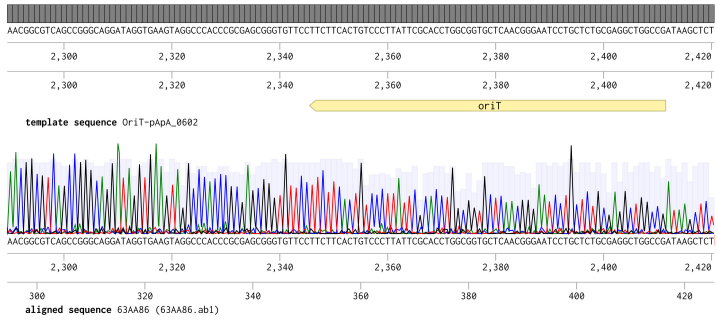


Figure 9.1: Alignment between template sequence of pAPA0602-oriT against sequencing result for its *oriT* region. A four-color chromatogram shows the results of the sequencing run. Base calls (bottom bases) match template (top bases) and some baseline noise can be seen. Visualization was achieved by alignment tool of Benchling (www.benchling.com).

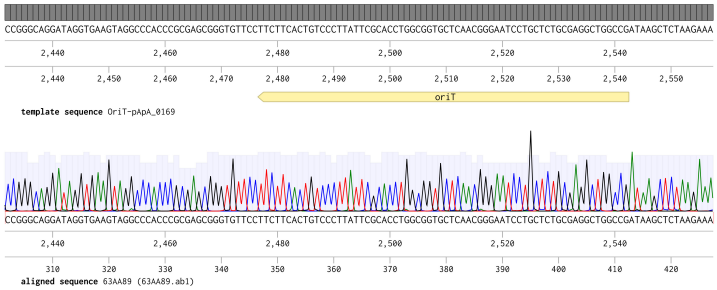


Figure 9.2: Alignment between template sequence of pAPA0169-oriT against sequencing result for its *oriT* region. A four-color chromatogram shows the results of the sequencing run. Base calls (bottom bases) match template (top bases). Visualization by Benchling (www.benchling.com).

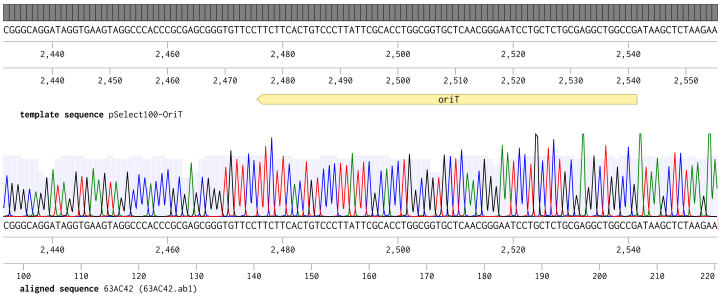


Figure 9.3: Alignment between template sequence of pSELECT100-oriT against sequencing result for its *oriT* region. A four-color chromatogram shows the results of the sequencing run. Base calls (bottom bases) match template (top bases). Visualization by Benchling (www.benchling.com).

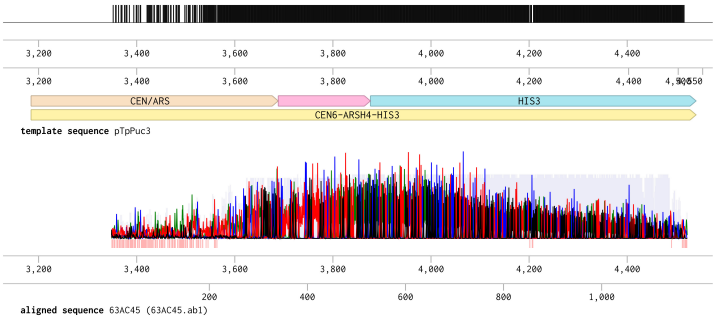


Figure 9.4: Alignment between template sequence of pAPA1001-YST against sequencing result for its *CEN6-ARSH4-HIS3* region. The alignment matches a large region in the middle of the inserted sequence. A mismatch appears around base 4200 on template axis. Red inverse peaks in the chromatogram indicate mismatch and noise. Visualization was achieved on alignment tool of Benchling (www.benchling.com).

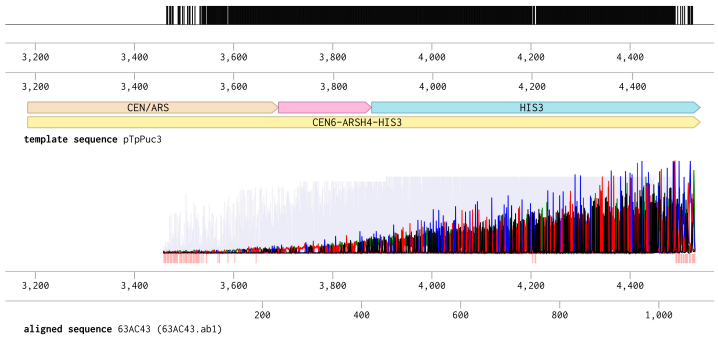


Figure 9.5: Alignment between template sequence of pAPA1001-YST against sequencing result for its *CEN6-ARSH4-HIS3* region. The alignment shows matching to template from the middle to the end of *HIS3* gene. A mismatch appears around base 4200 on template axis. Red inverse peaks in the chromatogram indicate mismatch and noise. Visualization was achieved by alignment tool of Benchling (www.benchling.com).

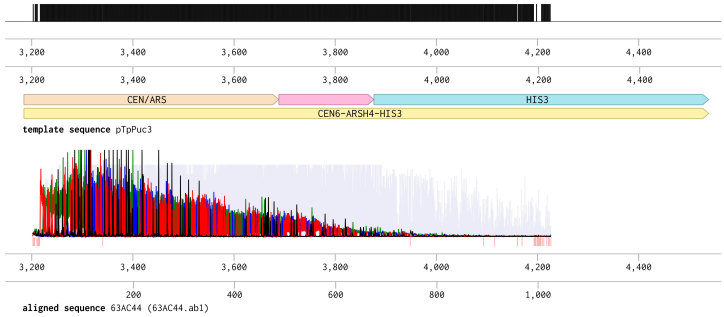


Figure 9.6: Alignment between template sequence of pAPA1001-YST against sequencing result for its *CEN6-ARSH4-HIS3* region. The alignment matches well starting from the *CEN/ARS* genes and becomes lower in quality as it reaches the middle. Red inverse peaks in the chromatogram indicate mismatch and noise. Visualization was achieved by alignment tool of Benchling (www.benchling.com).

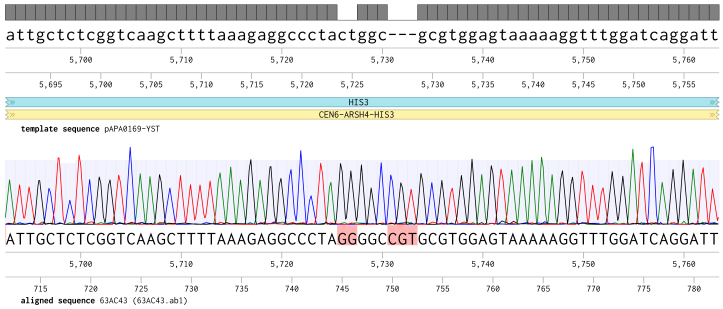


Figure 9.7: Mismatch in pAPA1001-YST constructs. The mutations are located in the *HIS3* gene of the cloned *CEN6-ARSH4-HIS3 sequence and consists of a conversion of AT to GG followed by an insertion of CGT. Visualization was achieved by alignment tool* of Benchling (www.benchling.com).

10 Conjugation

After cloning *oriT* and *CEN6-ARSH4-HIS3* sequences into the plasmids, a protocol for delivering the plasmid by bacterial conjugation to *Nannochloropsis* was established. In this chapter, the results of the transformations using the various constructs, donor strains and under varying conditions, are presented.

Functionality of cloned *oriT*

To test whether the cloned *oriT* was functional, a bacterial conjguation between *E. coli* S17-1 carrying pAPA0602-oriT and pAPA019-oriT and DH5α carrying pHH100 was performed. DH5α colonies were observed growing on the selective media containing both ampicillin and kanamycin, while control samples of *E. coli* S17-1 whereas DH5α were not able to grow on the selective media.

Compatibility between origins pUC and pBR2 with RK2 origin

The compatibility of the pAPA1001 (pUC origin, AmpR), pSELECT100 (pBR2 origin, AmpR) and pTA-Mob (pBBR1 origin, GenR) was tested by making recipient DH5α cells containing pHH100 (pBBR1 ori) electrocompetent and transforming them with pAPA1001-oriT and pSELECT100-oriT by electroporation. After overnight incubation on selective media containing both ampicillin and gentamycin, colonies on the plates were observed for all transformations. Refer to Section 12.3.2.

Evaluation of non-selective and selective media

Different co-incubation media were evaluated for the use of incubating both donor (*E. coli*) and recipient (*Nannochloropsis*) cells together. A trial conjugation experiment was performed and cells were plated together overnight on the different media. On the media containing the original *Nannochloropsis* media (enriched seawater), the *E. coli* were not able to grow, but *Nannochloropsis* growth was observed. On mixed media consisting of 50% LB media and 50% *Nannochloropsis* media, there was only a robust, white bacterial growth, typical of *E. coli* and no algal growth. Lastly, on the plates containing *Nannochloropsis* media with added 1% peptone, there an almost equal growth of both bacteria and algae. This can be clearly seen in Figure 10.1.

Selection plates were evaluated to determine which antibiotic is suitable for the elimination of *E. coli* after conjugation. Kanamycin was chosen because it does not inhibit the growth of *N. oceanica*, but eliminates *E. coli* [Chernyavskaya, 2014]. The selective plates were tested to ensure that wild type *N. oceanica* were not able to grow on them. No wild type growth was observed on both types of selective plates containing 20 µg/mL kanamycin and either 50 µg/mL hygromycin or 2 µg/mL zeocin.

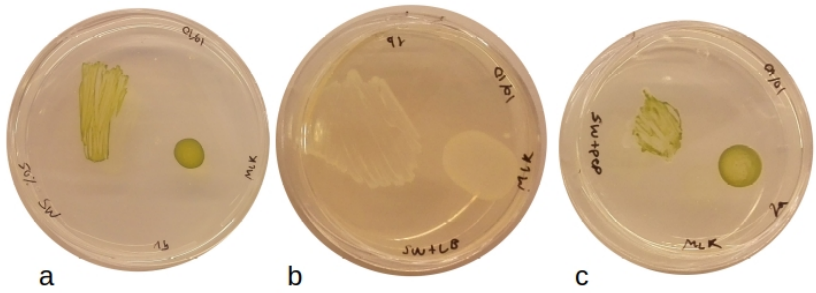


Figure 10.1: Growth on media types evaluated for the co-incubation of *E. coli* and *Nannochloropsis*. A conjugation mix consisting of *E. coli* S17-1 cells and *N. oceanica* was plated on three media types for evaluation: sea water enriched with Cell-Hi NC mediium (a), enriched sea water and LB media (b), enriched sea water and 1% peptone (c). Only algal growth present on (a), only bacterial growth on (b) and a mixed growth on (c).

Phenotype on plate

Conjugation was conducted on filter papers that could "hold" the cells, but allow contact with the media. After the initial co-incubation, the cells could then easily be transferred on to selective medium.

Initially,*Nannochloropsis*/*E. coli* conjugation cell mixtures appear green on the filter papers. However after the cells are transferred onto selective media, the algal cells slowly bleach out and no growth is observed. The plates were incubated under optimal conditions of constant light intensity at 76.5 µPAR and temperature of 23 °C. Colonies were generally observed within 14-21 days. After 7 days, colonies can be observed under a light microscope. Some colonies were not observed until after 4 weeks of incubation, while some were easily observed after 14 days. Figure 10.2 shows an colonies growing on the membrane filter paper. The round areas with raised borders within which the colonies are growing are where the alga/bacteria conjugation mixture was spotted on the membrane.

Figure 10.2: Phenotype of *N. oceanica* transconjugants growing on selection plate. Conjugation mixes of *E. coli* and *N. oceanica* cells were spotted in constant volumes on membrane filter papers laid on top of the solid media. A concentration of 2 µg/mL zeocin and 20 µg/mL kanamycin was used for selection and elimination of *E. coli*. Colonies appear after 2 weeks and reach this size after 4 weeks.

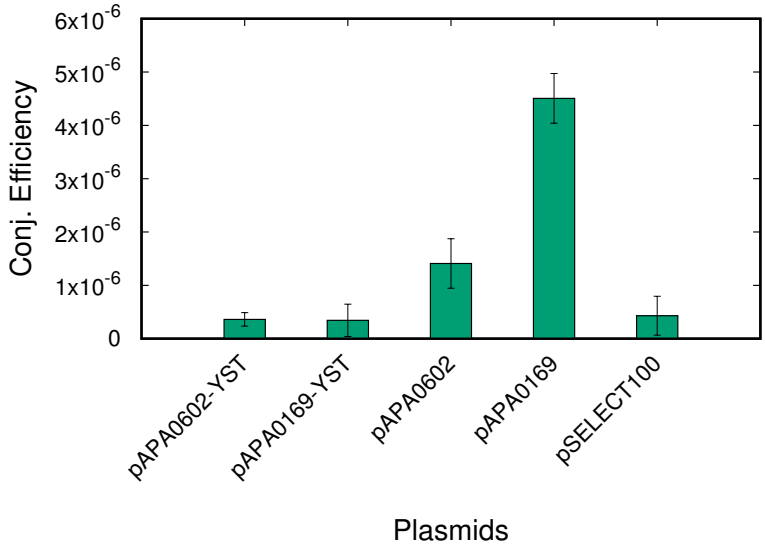


Figure 10.3: Average conjugation efficiencies of transfer of five plasmids to *N. oceanica* using *E. coli* DH10B/pTA-Mob as donor strain. The plasmids pAPA0602 (4576 bp), pAPA0169 (4787 bp), pAPA0602-YST (5928 bp), pAPA0169-YST (6059 bp) and pSELECT100 (4514 bp) were transferred via conjugation using a recipient-donor ratio of 1:2.5. The conjugation efficiency is calculated as the ratio of transconjugants by the number of recipient cells plated. The efficiency averages and standard deviations were calculated using at least three replicates.

10.1 Conjugation efficiency

Presented here are the results of the conjugation experiments which were performed according to protocol described in Section 6.1.2. Five constructs in total were used in the conjugation experiments: pAPA0602 (4576 bp), pAPA0169 (4787 bp), pSELECT100 (4514 bp), pAPA0602-YST (5928 bp) and pAPA0169-YST (6059 bp). All five constructs were introduced to both donor strains or *E. coli*, S17-1 and DH10B/pTA-Mob. The results show the conjugation efficiency, which represents the ratio between the number of transconjugants and the number of recipient cells plated.

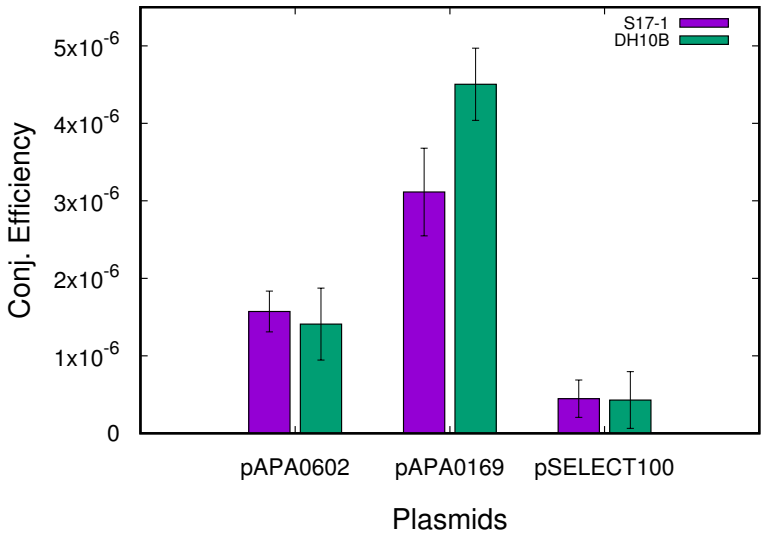


Figure 10.4: Comparison of average conjugation efficiencies of *E. coli* donor strains S17-1 (purple) and DH10B/pTA-Mob (green) for the transfer of pAPA0602 (4576 bp), pAPA0169 (4787 bp) and pSELECT100 (4514 bp) to *N. oceanica*. The plasmids were transferred via conjugation using a recipient-donor ratio of ∼1:2.5. The conjugation efficiency is calculated as the ratio of transconjugants by the number of recipient cells plated. The efficiency averages and standard deviations were calculated using at least three replicates.

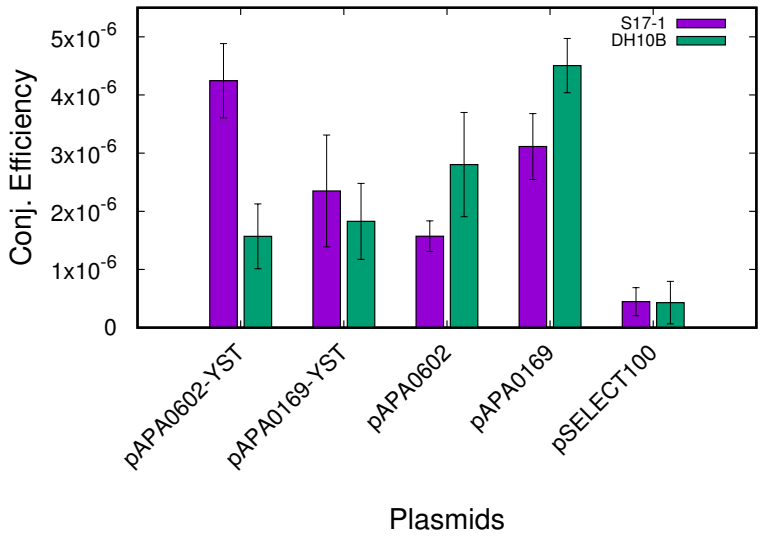


Figure 10.5: Comparison of best conjugation efficiencies achieved using *E. coli* donor strains S17-1 (purple) and DH10B/pTA-Mob (green) for the transfer of pAPA0602 (4576 bp), pAPA0169 (4787 bp), pAPA0602-YST (5928 bp), pAPA0169-YST (6059 bp) and pSELECT100 (4514 bp).The conjugation efficiency is calculated as the ratio of transconjugants by the number of recipient cells plated. The efficiency averages and standard deviations were calculated using at least three replicates.

Ratios were chosen based on previous literature, which states that increasing cell number in favor of the donor, results in higher higher efficiencies than applying the standard 1:1 ratio [Dominguez and O'Sullivan, 2013]. Many of the conducted experiments had a ratio between 1:2-1:7 (recipient:donor), before a ratio of 1:2-1:3 was chosen to be maintained for all future experiments. Due to favoring of *E. coli* strain DH10B/pTA-Mob during the last experimental period of the thesis, there is a lack of data for strain S17-1 at ratio of 1:2 - 1:3 for the plasmids pAPA0602-YST and pAPA0169-YST. A comparison of all five constructs under the same conditions (ratio 1:2.5) therefore only exists for the DH10B/pTA-Mob strain and is shown in Figure 10.3. However, in Figure 10.4, both strains are compared under same conditions for three of the plasmids (pAPA0602, pAPA0169 and pSELECT100). The highest efficiencies for all plasmids obtained by both strains

Table 10.1: The highest efficiencies obtained using S17-1 and DH10B/pTA-Mob strains and the ratios used.

Plasmid	**S17-1**	**ratio**	**DH10B**	**ratio**
pAPA0602-YST	$4.2 * 10^{-6}$	1:5.8	$1.57 * 10^{-6}$	1:7
pAPA0169-YST	$2.3 * 10^{-6}$	1:5.5	$1.8 * 10^{-6}$	1:6
pAPA0602	$1.6 * 10^{-6}$	1:2.8	$2.8 * 10^{-6}$	1:4
pAPA0169	$3.1 * 10^{-6}$	1:3	$4.5 * 10^{-6}$	1:2.5
pSELECT100	$4.5 * 10^{-7}$	1:2	$4.3 * 10^{-7}$	1:2.5

are presented in Figure 10.5. The ratios and other data that produced the best efficiencies are shown in Table 10.1.

We can rely only on the results of Figure 10.3 to determine which plasmid was more successful, because the conjugations were conducted using the same recipient to donor ratio (1:2.5). pAPA0169, resulting in conjugation efficiency $\sim$4.5 x 10^{-6} is therefore the most successful. pAPA0602 comes second with an efficiency at $\sim$1.4 x 10^{-6}, followed by pSELECT100 ($\sim$4.3 x 10^{-7}), pAPA0602-YST ($\sim$3.6 x 10^{-7}), and lastly pAPA0169-YST ($\sim$3.4 x 10^{-7}). Based on the graph in Figure 10.4, there is no significant difference in conjugation efficiency between the two donor strains except for pAPA016 where *E. coli* DH10B/pTA-Mob appears to result in higher conjugation efficiency. For pSELECT100 and pAPA0602, strain S17-1 results in slightly higher conjugation efficiency. As can be seen in Figure 10.5, the highest efficiency is achieved with pAPA0169 (strain DH10B/pTA-Mob).

Strain S17-1 shows higher conjugation efficiencies for pAPA0602-YST and pAPA0169-YST (Figure 10.5), however at a different recipient to donor ratio that what is presented for the other plasmids. It is important to note the data presented in Table 10.1, because the recipient to donor ratio is an important factor on the conjugation efficiency.

10.2 Optimization of conjugation protocol

For optimizing the conjugation protocol, *E. coli* DH10B/pTA-Mob was chosen over S17-1 as donor strain. It has been reported to give more reliable results when compared to S17-1 or SM10 strains that can introduce mutations in the mobilized DNA [Strand et al., 2014]. The experiments were also conducted using only the pAPA0169 plasmid. The results here are again presented as the conjugation efficiency.

10.2.1 Optimizing recipient to donor ratio

In a ratio optimization experiment, the ratios between donor and recipient cells were tested (Section 6.3.1, Table 6.1) to find ratios where the highest conjugation efficiency can b observed. Ratio optimization result, presented in Figure 10.6 are contrasting with the trend shown in Table 10.1 where the highest efficiencies were achieved with higher donor number relative to recipient. In the ratio optimization experiment, the efficiency increases almost linearly with the ratio favoring the recipient cell number, with the highest efficiency (1.5×10^{-6}) achieved at a ratio of 4:1 (recipient to donor).

10.2.2 Optimizing co-incubation conditions

For optimization of time in the protocol, incubation times at 30 °C of the conjugation mixtures were varied from the standard protocol of 90 minutes. Incubations were performed at 10, 30, 90 and 180 minutes both in the dark as well as under light. The efficiencies achieved for incubations under light conditions are presented in Figure 10.7a, and in dark in Figure 10.7b.

Transconjugants were obtained even for the shortest incubation time of 10 minutes. For both light and dark incubations, there is a similar trend where the conjugation efficiency peaks at 90 minutes. Comparing the two light conditions in Figure 10.7c, the dark incubations show a higher efficiency except for at 10 minutes. Hence, the highest efficiency, at 4.5×10^{-6}, was achieved for incubation at 90 minutes in the dark. It should be noted however, that the comparisons are made using the average efficiencies and the standard deviation for 90 minutes under light (seen in Figure 10.7a) is quite large.

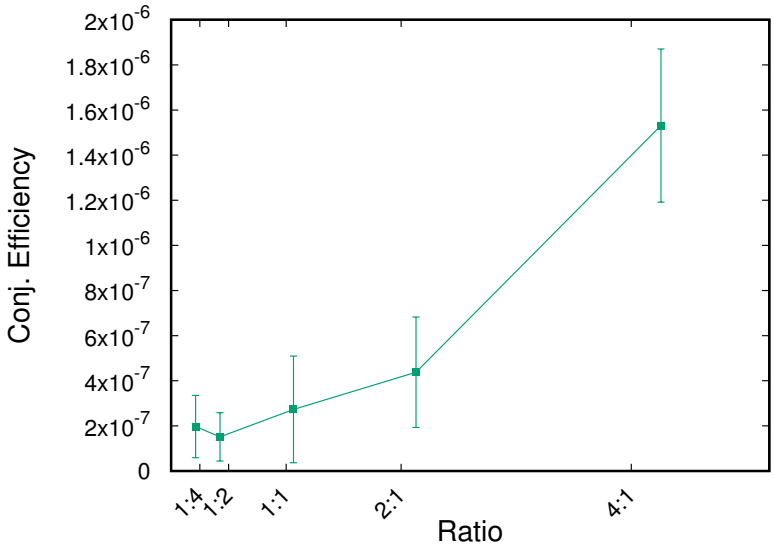
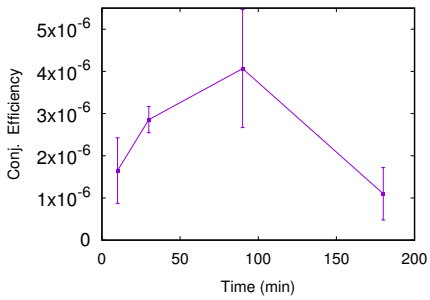
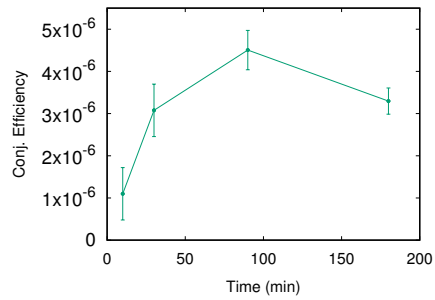


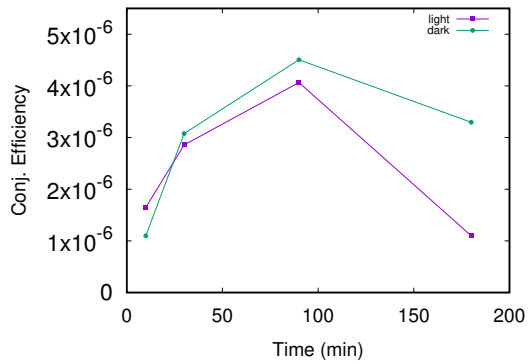
Figure 10.6: Average conjugation efficiencies at varying recipient:donor ratios. The ratios 4:1, 2:1, 1:1, 1:2 and 4:1 were tested on the conjugation efficiency using donor strain *E.coli* DH10B/pTA-Mob to transfer pAPA0169 to *N. oceanica*. The conjugation efficiency is calculated as the ratio of transconjugants by the number of recipient cells plated. The efficiency averages and standard deviations were calculated using at least three replicates.



(a) Light



(b) Dark



(c) Light vs dark

Figure 10.7: Comparison of conjugation efficiencies for varying co-incubation times in dark (green) and light (purple) conditions. Incubation periods of 10, 30, 90 and 180 minutes were tested on the conjugation efficiency using donor strain *E.coli* DH10B/pTA-Mob transferring pAPA0169 to *N. oceanica*. Efficiency peaks at 90 minutes for both light and dark. The conjugation efficiency is calculated as the ratio of transconjugants by the number of recipient cells plated.

11 Electroporation and comparison with conjugation

Following linearization of the five plasmids, pAPA0602 (4576 bp), pAPA0169 (4787 bp), pSELECT100 (4514 bp), pAPA0602-YST (5928 bp) and pAPA0169-YST (6059 bp), confirmation of plasmid size and linearization was achieved by gel electrophoresis of which the results are shown in Figure 11.1. Concentration of the plasmids that were applied can be seen in Table 7.1. Electroporation was performed according to methods described in section 7.1. The amount of DNA and cells used in the electroporations are presented in Table 7.1 and the results are presented as the transformation efficiency of electroporation (cfu/cell/µg plasmid DNA).

Colonies appeared after 10-14 days, but more continued to appear up to 4 weeks after transformations. The transformation efficiencies achieved for each construct is presented in Figure 11.2. It is clear from the graph that pAPA0602 resulted in significantly higher transformation efficiency compared to the other plasmids, with an efficiency of $\sim$3.7 x 10^{-6} cfu/cell/µg plasmid DNA. That is more than double than the next highest efficiency ($\sim$1.4 x 10^{-6} cfu/cell/µg DNA) achieved using pAPA0169. The remaining three plasmid, pAPA0602-YST, pAPA0169-YST and pSELECT100 appear to have given similar efficiencies, all lying around $\sim$4.3 x 10^{-7} cfu/cell/µg DNA.

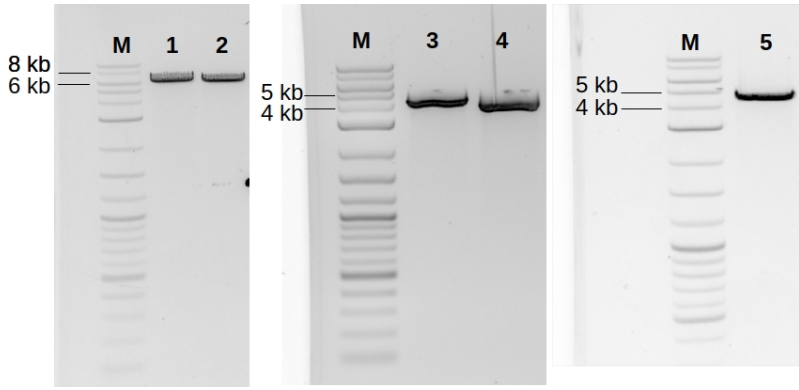


Figure 11.1: Linearized plasmids visualized by gel electrophoresis. 1, pAPA0169-YST (6059 bp); 2, pAPA0602-YST (5928 bp); 3, pAPA0169 (4787 bp); 4, pAPA0602 (4576 bp); 5, pSELECT100 (4514 bp). M: NEB's 2-log ladder marker.

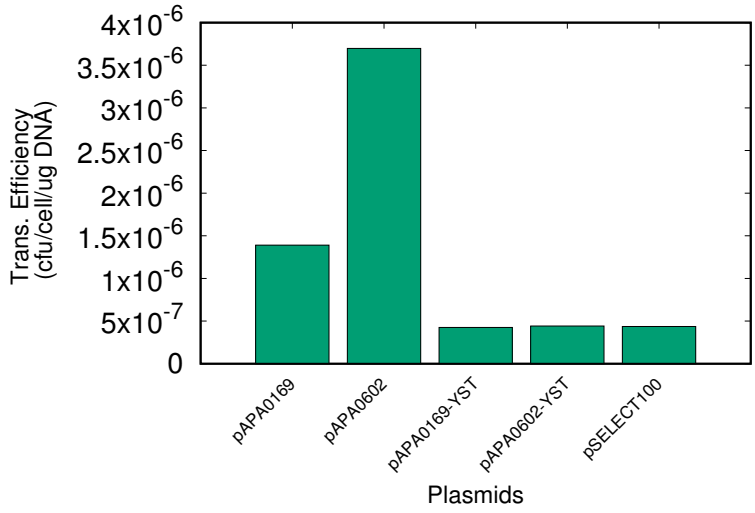


Figure 11.2: Transformation efficiency (cfu/cell/µg DNA) of *N. oceanica* for the linearized plasmids pAPA0602, pAPA0169, pAPA0602-YST, pAPA0169-YST and pSELECT100.

11.1 Comparison with conjugation

Comparison between the highest efficiencies obtained by electroporation with the highest from conjugation can be seen in Figure 11.3. The best efficiencies obtained from conjugation are presented for both *E. coli* donor strains (DH10B/pTA-Mob and S17-1).

The highest efficiencies were obtained by conjugation, except for the plasmid pAPA0602, which resulted in higher transformation ($\sim$3.7 x 10^{-6} cfu/cell/µg DNA) efficiency by electroporation. Generally, electroporation seems to have resulted in lower efficiencies compared with conjugation, except for pSELECT100 where both methods, regardless of donor strain, resulted in low efficiencies when compared with pAPA1001 constructs.

11.2 Evaluation of pAPA1001 promoters with zeocinR as reporter

The tolerance to zeocin conferred by the *N. oceanica* endogenous promoters (nanno-602 and nanno-169) of pAPA0602 and pAPA0169 was evaluated. *Nannochloropsis* was transformated by electroproation with the linearized plasmids and then plated on selective media containing increasing antibiotic concentration. The zeocin concentrations used were 2, 4, 8 and 16 µg/mL. The results of the experiment are presented as the relationship between transformation efficiency and concentration of the antibiotic. The results for both plasmids are combined in Figure 11.4.

A similar pattern is apparent for both plasmids where the efficiency is relatively high at 2 µg/mL, decreasing substantially at 4 µg/mL followed by further gradual decrease towards 8 and 16 µg/mL (Figure 11.4). It is clear from the graphs that higher transformation efficiencies were obtained with pAPA0602.

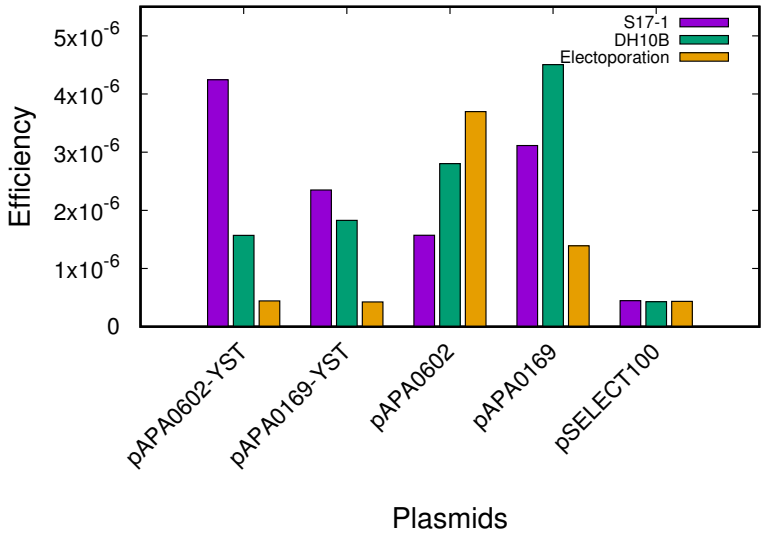


Figure 11.3: Comparison of best transformation and conjugation efficiencies obtained for all plasmids is shown. The transformation efficiency achieved by electroporation (orange) is presented as cfu/cell/µg plasmid DNA. The conjugation efficiencies using donor strains *E. coli* S17-1 (purple) and DH10B/pTA-Mob (green) are presented as the ratio of transconjugants by the number of recipient cells plated.

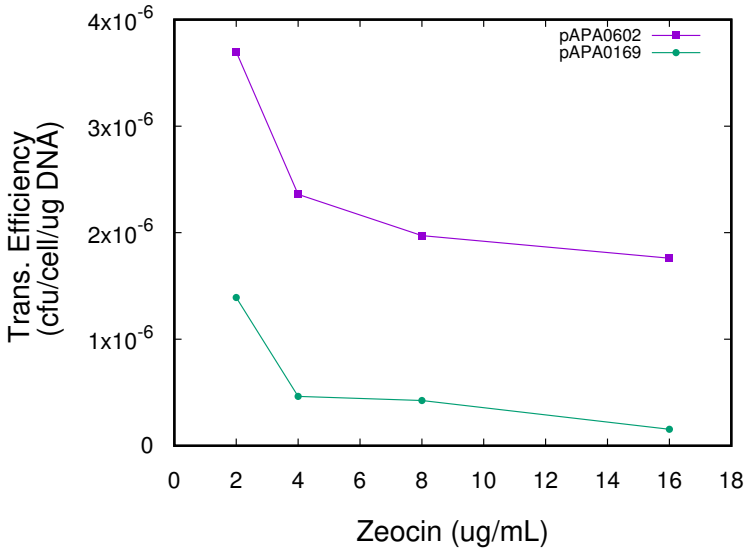


Figure 11.4: Tolerance of *N. oceanica* to zeocin conferred by the promoters nanno-169 (pAPA0169) and nanno-602 (pAPA0602) presented as transformation efficiency (cfu/cell/µg plasmid DNA) per µg/mL zeocin.

Part IV

Discussion

12 Discussion

12.1 Growth optimization

An improved growth medium was developed, which provides faster and enhanced growth. For this, five different media were evaluated: artificial sea water (ASW) enriched with Cell-Hi NC medium, filtered sea water (FSW) enriched with Cell-Hi NC medium, FSW enriched with F/2 medium, non-enriched FSW and non-enriched ASW. The growth of *N. oceanica* was followed by imaging and growth rates were calculated by a script. The calculated relative densities were then analyzed to create growth curves.

Non-enriched media were not included in the analysis because cells bleached out. This was probably due to the absence of vitamins and vital nutrients that the alga needs for its metabolism and growth. The constituents of Varicon's Cell-Hi NC medium is unknown, but F/2 medium contains among other components the vitamins biotin (B7), cyanocobalamin (B12) and thiamine (B1) (Table A.1). Vitamins are known to form cofactors important for cellular functions and the importance of biotin, thiamine and cobalamine to vitamin autotrophic algae has been extensively reported [Croft et al., 2006]. Observing no growth and photobleaching in absence of F/2, indicates that its vitamins and minerals are vital. Since no growth was observed on medium lacking Cell-Hi NC, we can assume that this medium supplement also contains vitamins and nutrients that are crucial for the growth of *Nannochloropsis*.

The reported growth curves (Figure 8.1) include the logarithmic growth phase, but lack the stationary growth phase. This means that the experiment, conducted over 14 days, should have proceeded for another week or two. The new media

established in this project, consisting of ASW enriched with Cell-Hi NC medium and resulted in improved growth when compared with the other media. When comparing FSW with ASW, which have both been supplemented with Cell-Hi NC, we see that ASW plays an additional role in improving the growth. Since we do not exactly know the constituents of Cell-Hi NC or Instant ocean, the commercial powder used to make ASW, this improvement can not be explained.

12.2 Molecular cloning methods

Three plasmids, pSELECT100, pAPA0169 and pAPA0602 were altered by insertion of *oriT* that renders them mobile for conjugal transfer and some were altered further to include the *CEN6-ARSH4-HIS3* sequence. Two different cloning methods were used in this project. Both methods were successful and no complications were encountered, however there are differences to two methods.

For cloning of *oriT*, digestion-ligation cloning was applied where the insert and backbone fragments are digested using the same enzyme (SapI or KpnI) to produce complementary ends that are then ligated by ligase. Cloning of the *CEN6-ARSH4-HIS3* sequence was accomplished with Gibson cloning.

Gibson cloning is a seamless cloning technique which relies on the action of several enzymes (ligase, exonuclease and polymerase) suspended in one reaction, which also requires specific buffers. As kits for Gibson cloning were used, this way of constructing vectors has more material costs than conventional digestion-ligation cloning. Gibson also requires much longer sticky ends than restriction enzyme digestion does. Gibson cloning has the additional advantage of assembling several fragments together in one reaction. For this project however, only two fragments at a time were assembled. Digestion-ligation cloning where ligation of fragments is performed in separate reactions, is more costly time-wise when compared to ligation occurring simultaneous with overhang generation in the Gibson assembly. Hence for this project, Gibson and digestion-ligation cloning took equal periods of time to complete. A limitation to digestion-ligation cloning which Gibson cloning does not have, is the necessity to find an appropriate restriction site to fit the insert fragment. This was not encountered as a significant problem in the project.

12.2.1 Confirmation by sequencing

After running a colony PCR and restrictive digestions to confirm insertion of the *oriT* region in the pAPA1001 and pSELECT100 plasmids, samples were sent for sequencing. Sequencing primers were designed to bind approximately 100 bp outside the region of interest due to the common occurrence of low resolution calls and noise in the beginning of a read. Only one sequencing sample containing one primer was sent for sequencing because the region of interest was relatively short, (under 500 bp). The sequencing results for *oriT*, which can be seen in Figures 9.1, 9.2 and 9.3, show a clear match when aligned against the sequence of *oriT*. The chromatogram peaks are mostly evenly separated and with minimal baseline noise, indicating a reliable read. The cloning of *oriT* was therefore considered a success.

Sequencing of the inserted *CEN6-ARSH4-HIS3* sequence, which is approximately 1.4 kb long, involved sending several samples in effort to cover the entire region of interest. The primers used for sequencing *CEN6-ARSH4-HIS3* were not specifically designed for sequencing, but were rather the same forward and reverse primers used to amplify the region during Gibson cloning. Therefore, noise and inaccuracy were expected for the start of the reads, which are within the region of interest. When viewing all three alignments (Figures 9.4, 9.5 and 9.6), there is clear overlapping within the cloned DNA sequence, indicating that the correct sequence was indeed inserted.

As mentioned in the results, there appears to be some mismatch on closer inspection (Figure 9.7). The mismatch appears in the *HIS3* gene of the sequence and is a conversion from CT to GG followed by an insertion of CGT. The conversion and the insertion are three bases apart. Because the inserted fragment was amplified using PCR, the DNA polymerase could have introduced those mutations [McInerney et al., 2014]. Another possibility is that the pPtPuc3 plasmid received and which was used as a template for amplification of the CEN6-ARSH4-HIS3 sequence, already contained those mutations in the *HIS3* region.

Such mutations could affect the protein-coding sequence. A frameshift mutation caused by the insertion of deletion of one or two nucleotides can have severe effects on the protein by changing the reading frame entirely. Insertion or deletion of three nucleotides does not cause the frame to shift and might have little or no effect on the protein. In this case the insertion of CGT results in arginine being added to the protein sequence. A conversion does not cause a frameshift

either, but changes the codon(s). The conversion of CT to GG changed a codon from CTG to GGG. This translates to a change from leucine to glycine. This is a missense mutation that might or might not have implications for the protein. Since HIS3 encodes a protein catalyzing histidine biosynthesis for yeast, it is not clear what role this gene has in maintaining the plasmid as an episome in the host. As mentioned in Chapter 3, it is the *CEN6* and *ARSH4* genes that have a low GC content typical of protist centromeres.

If the mutations indeed cause a disturbance to the episomal functions of the sequence, then a reduced transformation or conjugation efficiency is expected compared to plasmids that do not contain *CEN6-ARSH4-HIS3*. A similar efficiency is expected. If the mutations have no effect on the episomal functions, a higher efficiency might be expected due to the plasmid being maintained in *Nannochloropsis*, leading to an increased probability that the zeocin resistance gene is integrated in the genome.

12.3 Conjugation

12.3.1 Media evaluation

Three types of non-selective media for the purpose of incubating both donor (*E. coli*) and recipient (*N. oceanica*) cells together were prepared for evaluation. A media that can sustain the growth of both organisms until selection was desired. The results are presented in Chapter 10, Figure 10.1.

The media containing half LB media and half *Nannochloropsis* media (enriched 50% SW) resulted in only *E. coli* growth and absence of algal growth. Possibly the use of 50% LB media diluted the *Nannochloropsis* media to an extent where the nutrient level was insufficient for algal growth. The LB media mixed with enriched SW, created an environment favorable for *E. coli* growth which outcompeted the algae and this media was determined as unsuitable for the desired purpose. The plating media containing only *Nannochloropsis* media was also considered unsuitable because in the same it sustained the growth of only one organism, the algae. Finally, the media containing *Nannochloropsis* media with added 1% peptone was able to sustain both organisms. Peptone provides *E. coli* with a complex mixture of peptides resembling nutrients found in the natural environment and has been shown to help *E. coli* growth in high-osmolarity

medium such as sea water [Amezaga and Booth, 1999].

12.3.2 Testing *OriT* function and origin compatibility

The compatibility of the pAPA1001 (pUC origin, AmpR), pSELECT100 (pBR2 origin, AmpR) and pTA-Mob (pBBR1 origin, Gm^r) was tested by making recipient DH5α cells containing pHH100 (pBBR1 origin, KanR) electro-competent and transforming them with pAPA0602, pAOA0169 and pSELECT100 by electroporation. Transformants were obtained for all combinations, indicating that the cells were able to maintain both pHH100 and pAPA1001's or pSELECT100. The origins are therefore compatible and no problems should be experienced with regard to the maintenance of a two-plasmid system in the donor strain DH10B/pTA-Mob.

To test whether the cloned *oriT* was functional, a bacterial conjugation between *E. coli* S17-1 carrying pAPA0602 and pAPA0169 and DH5α carrying pHH100 was performed. DH5α colonies were observed growing on the selective media containing both ampicillin and kanamycin, while control samples of *E. coli* S17-1 and DH5α were not able to grow on the selective media. This indicates that there was a successful conjugation event and that the cloned *oriT* is functional. By sequencing the cloned region it was again confirmed that indeed the correct sequence of *oriT* was inserted.

12.3.3 Establishing and optimizing conjugation protocol

The established protocol described in Section 6.1.2 was based on the protocol use in the study by Karas et al. (2015) as well as a standard protocol for bacterial conjugation, but was further modified in order to increase the number of transconjugatns [Karas et al., 2015]. As mentioned, in the preliminary work (section 6.4), the crucial step of 90 minute incubation at 30 °C was not yet applied and various steps in the protocol were developed based on favorable conditions for mediating conjugation and retaining the viability of *N. oceanica*.

The project-specific parameters and conditions that were established for the conjugation protocol included using a ratio of recipient to donor cells that was higher in number of donor cells. Additionally, the ratio parameter was also evaluated by testing different ratios described in Figure 10.6. Incubation time

for the conjugation mixture after the 90 minute 30 °C incubation step, but prior to selection varied and further evaluation of this parameter was necessary as well (Figure 10.7c). In a previous study, reported incubation times for conjugation for the heterokont algae *P. tricornutum* was 2 days, while *T. pseudonana* was only incubated for 4 hours. A time in between those mentioned was chosen for the incubation of *N. oceanica* in this project, being an overnight incubation (12-16 hours). The temperature (23 °C) was chosen in this project as it is the optimal growth temperature for *N. oceanica*. Another modification to existing protocols for the conjugation involving heterokont algae as recipients is the use of membrane filter papers. Membrane filter papers were chosen for their ease of use for transferring the cells onto selective media. Using membrane filters to hold the cells also reduced the loss of and perhaps stress on cells when compared to scarping the cells off the plate and resuspending them.

There are additional conditions used in previous protocols [Karas et al., 2015], that were considered important and therefore were included in the protocol, established in this project. These include the use of equal volumes (not number) of donor and recipient cells to maintain equal conditions for both with regard to the medium. The most critical step however, is the 90 minute incubation at30 °C. Without this incubation step, no conjugation was achieved. The reason for the efficiency of this step is probably that conjugation is a temperature-sensitive process for the bacteria. The temperature optimum of the incubation step appears rather specific, as in preliminary work, temperatures up to 27 °C were tested, but did not result in the detection of conjugation. With the optimum temperature for *E. coli* being 37 °C, 30 °C could be the threshold for such activity to take place. Another reason for the temperature requirement could be that the membranes of *Nannochloropsis* become more permeable by higher temperature [Fettiplace and Haydon, 1980]. Additionally, there might be a defense mechanism for identifying and degrading foreign DNA in the algae that does not work at higher temperatures, but this needs to be investigated further for confirmation.

Light and dark incubations

To further increase the number of transconjugants, it was evaluated how the incubation times under light and dark conditions affect conjugation efficiency.

The results shown in Figure 10.7c, were very successful in defining efficient co-incubation conditions and insights into the conjugation process. It can now be assumed that it takes as little as 10 minutes to achieve conjugation. Incubation

in the dark achieved higher efficiencies. However transconjugants were obtained from incubation under light as well, meaning that dark conditions are not necessary for conjugation to occur. The efficiency for conjugation in the dark and in the light increase, peaking at 90 minutes and then decrease again. The increase in conjugation efficiency is nearly linear up to the detected maximum (90 minutes). The decrease in efficiency at 180 minutes could be the result of *N. oceanica* not surviving longer incubations at 30 °C, which could easily be tested by exposure to higher temperatures and assessment of survival.

The developed protocol retained the overnight incubation under favorable conditions reported for the conjugation of other heterokont algae [Karas et al., 2015]. For *N. oceanica* a temperature of 23 °C under light (76.5 µPAR) were chosen to provide an acclimatization period that the algae might need after the stress imposed on it from handling (centrifugation, resuspension) and perhaps also from conjugation. A resting period is also a usual step for heat shock transformation and electroporation protocols.

Ratio optimization

The results for optimizing the ratio between recipient and donor were contrary to what was expected, which was that increasing donor cell numbers would result in higher efficiency. Figure 10.6 shows clearly a trend for the ratios, but in the opposite direction, increasing efficiency as recipient (algal) cell numbers increase in the ratio. This result could be due to an increase of false positives created by the growth of algal cells as satellite colonies on top of other cells, shielding the cells from contact with the selection antibiotic. This hypothesis can be examined by repeating the experiment while using smaller cell numbers to eliminate the risk of satellite colonies. Although a correlation between efficiency and ratio in favor of recipient cell is seen in Figure 10.6 the efficiency achieved was not higher than that achieved by a ratio in favor of donor cells (Figure 10.5. This complex issue depends on the concentration and total number of the donor and recipient cells, and their interaction. This project has shown that ratio optimization needs further research to find the most suitable ratios.

12.3.4 Conjugation results

Ideally, an optimization of protocol is performed before applying the most effective conditions in all further experiments. However, in this project optimization of the conjugation protocol could only have been accomplished after a critical step in the conjugation protocol was established.

Conjugation efficiency was calculated for the transfer of the five constructs pAPA0169, pAPA0602, pAPA0169-YST, pAPA0602-YST and pSELECT100. The highest efficiency was achieved using pAPA0169 when compared to the other plasmids under similar conditions (Figure 10.3). The pAPA0169-YST and pAPA0602-YST constructs which include the *CEN6-ARSH4-HIS3* sequence gave lower efficiencies than their pAPA0169 and pAPA0602 counterparts for the strain DH10B/pTA-Mob. However, for the strain S17-1, one of the highest efficiencies was obtained using pAPA0602-YST (Figure 10.5). These findings are contradicting and not much can be concluded about the role of *CEN6-ARSH4-HIS3* in the improvement of conjugation efficiency. Further investigation could reveal the effect of *CEN6-ARSH4-HIS3* on conjugation efficiency. Nevertheless, using the original plasmids, satisfactory efficiencies were obtained.

***E. coli* S17-1 vs. DH10B/pTA-Mob**

Donor strains *E. coli* S17-1 and DH10B/pTA-Mob showed no significant difference in achieved conjugation efficiency (Figure 10.4). Although the protocol for using either strains is the same, it is worth noting that the donor strains are established differently. *E. coli* S17-1 are competent cells that can be made into donors by heat shock transformation. The DH10B/pTA-Mob strain contains the mobilization plasmid and therefore needs to made electro-competent and then transformed with a cargo plasmid by electroporation. The established donor strain DH10B/pTA-Mob also needs to be maintained under selection of two antibiotics to ensure that both plasmids are present. The procedure for establishing DH10B/pTA-Mob as donor is therefore both more time consuming (more steps) and expensive (several antibiotics, electroporation cuvettes and system) compared to S17-1. Another drawback to using DH10B/pTA-Mob is that the selection marker for pTA-Mob is gentamicin, a relatively hazardous antibiotic to work with [National Center for Biotechnology Information, PubChem Compound Database; CID=3467]. The drawbacks to working with DH10B/pTA-Mob compared to S17-1 are balanced by the fact that it mediates conjugation without

causing mutations or inserting donor DNA during mobilization.

Possible errors

The antibiotic zeocin is known to degrade over time and especially with exposure to light as it is light-sensitive [Invitrogen, (www.thermofisher.com)]. This is a plausible source of error in this project since selective media containing zeocin was placed both under constant light and for extended periods due to the growth requirements of *N. oceanica*.

Replicates used in this project to generate averages and standard deviations are what can be called "pseudoreplicates" [Ruxton and Colegrave, 2011]. That means that the data collected from each "point" is not statistically independent, because the same conjugation mixture is used to make several plates or spots on the membrane filter papers. The implication of this, is that statistical tests such as the t-test and analysis of variance (ANOVA) which rely on independent errors can not be applied, as correlated errors lead to false precision and p-values that are too small [Lazic, 2010]. For the data to be statistically independent, different cultures would have to be used to create the replicates. Practically, this means at least three different bacterial cultures (per donor strain, per plasmid) and three different algal cultures would have to be used per experiment.

Another source of error in reporting the data may be the lack of confirming transconjugants. Performing this confirmation could be achieved by colony PCR, thus assessing false positives or giving a percentage of confirmed transconjugants that then can be extrapolated onto other results. The reason colony PCRs were not performed in this project was due to time constraints. There are five constructs and a large number of colonies would have been picked to create a statistically reliable result. This in itself is not the most time consuming procedure, but because *N. oceanica* and *E. coli* were moved together (on membrane papers) onto selective media, the *E. coli* cells were killed, but *E. coli* DNA remained present around the newly formed transconjugants, this would have introduced bias in the colony PCR results. The way to solve this is by transferring transconjugants on to master plates and waiting for new algal colonies to form before performing colony PCRs. There was not enough time during the thesis period to grow colonies of *N. oceanica* and assess the number of transconjugants by colony PCR.

How can it then be certainly known that conjugation took place and created

transconjugants? Unless the transconjugant colonies are all false positives created by satellite colonies, there is actually no other way the zeocin resistant colonies could have formed. A clear argument that the transconjugatns are not false positives arises from the preliminary work of this thesis. Lacking the incubation step, no algal growth was observed. The effectiveness of the antibiotics have been determined in the past [Kilian et al., 2011]. There is therefore no reason to doubt the success of transkingdom conjugation achieved between *E. coli* and *N. oceanica*.

12.4 Electroporation

Electroporation was performed as a control method for comparison with conjugal transfer. The conjugation and transformation efficiencies were compared for all of the five constructs (pAPA0602, pAPA0169, pAPA0602-YST, pAPA0169-YST and pSELECT100).

After linearization of the the constructs, they were used to transform *N. oceanica* and the results are presented in Figure 11.2. pAPA0602 resulted in the highest ($\sim$3.7 x 10^{-6} cfu/cell/µg plasmid DNA) transformation efficiency, followed by pAPA0169 ($\sim$1.4 x 10^{-6} cfu/cell/µg DNA). Electroporation with the pAPA0602-YST, pAPA0169-YST and pSELECT100 plasmids had efficiencies around $\sim$4.3 x 10^{-7} cfu/cell/µg DNA. The transformation efficiencies achieved by electroporation for pAPA0169 and pAPA0602 are higher by two orders of magnitude compared to those previously achieved (1.2x10^{-8} and 6.5x10^{-8} colonies/cell/µg DNA) for the plasmids [Nguyen, 2016].

In Table 7.1, the amount of the various plasmids and number of cells used in the electroporation are described. Knowing that there is a linear relationship between amount of DNA and number of transformants [Kilian et al., 2011], large amounts of DNA (2-3 µg) were used in an effort to get the highest number of transformants. Since the transformation efficiency is calculated by dividing transformants per µg DNA, the differences in amounts of DNA used are accounted for. The difference in amount of plasmid used between pAPA0602 and pAPA0169 is about 10%, yet the transformation efficiency achieved with pAPA0602 is more than double that of pAPA0169. This suggests that a factor, other than amount of DNA, could be responsible for the higher efficiency achieved in pAPA0602.

12.4.1 Evaluation of pAPA1001 promoters

Methods to study promoter activity are commonly based in the expression of a reporter gene from the promoter of the gene of interest. In this study a zeocin resistance conferring gene was placed under control of two promoters, thus acting as a reporter gene, whose expression can be assessed by investigating the growth characteristics on different concentrations of zeocin. For the pAPA0169 and pAPA0602 plasmids, the endogenous promoters *nanno-169* and *nanno-602*, expression levels are described as being more stable and higher than previously used promoters, such as the LDSP promoter of pSELECT100. The evaluation of the plasmids was based on the tolerance to zeocin conferred by the promoters on *N. oceanica*.

Figure 11.4 shows a comparison between the tolerance of *N. oceanica* transformed by pAPA0602 vs pAPA0169 as efficiency (cfu/cell/µg DNA) per zeocin concentration (µg/mL). Although pAPA0602 resulted in higher efficiencies, both plasmids confer the same pattern of tolerance to zeocin where there is a relatively high tolerance at 2 µg/mL zeocin which decreases greatly at 4 µg/mL and then slightly more towards 8 and 16 mug/mL zeocin. As both promoters provide similar level of zeocin resistance, they may express the resistance conferring gene at similar levels. In the future, a zeocin concetration of 4 µg/mL can be chosen for selection to minimize false positive clones arising from satellite colonies.

12.5 Disadvantages and advantages of conjugation over electroporation

Comparing electroporation with conjugation, both methods have disadvantages and advantages over the other. Based on the highest achieved efficiencies on generating strains containing transferred DNA, shown in Figure 11.3, conjugation appears the more efficient method for delivery of the constructs.

If the tools necessary for a conjugation protocol (established donor strain) are available, performing conjugation-mediated DNA transfer is not a challenging process. However, if the donor strain is not available and has to be established first, this method of plasmid delivery can be considered unnecessarily laborious. Plasmids that are to be transferred must include *oriT* to enable the plasmid to be transferred during conjugation. The *oriT* must be confirmed by sequencing

and by testing its function. However, some plasmids already have *oriT* and do not need further modification. Donor strains need to be made and can either involve S17-1 or a two plasmid system such as the one created by pTA-Mob. Either strain needs to be grown and transformed, one by heat shock, which is rather easy and fast, the other by electroporation, which is also easy, but more time consuming. Confirmation of donor colonies carrying the plasmid is necessary before the donor can be used in conjugation. A conjugation experiment needs to be planned in advance so that overnight cultures are ready ahead of time. It takes time for the 1% cultures of the donor strains to achieve the desired optical density before cells are collected for conjugation. If a certain ratio is desired, cell concentration present in the cultures and volumes needed must be calculated after measuring the optical density. Incubation first at 30 °C for 90 minutes is then followed by transfer to conditions favorable to the recipient with overnight incubation. The following day, selection can be made by transferring cells to selective media and incubating further.

If the most straightforward method is heat shock transformation (HST) then electroporation lies in between HST and conjugal transfer of DNA. Electroporation involves linearization and concentrated of DNA to a desired amount, before recipient cells are washed multiple times and then electroporated. The cells need then a resting period, usually an overnight incubation. This is then followed by selection. Colony PCR is much easier to perform on electroporated *Nannochloropsis* as colonies can be transferred to master plates and used in the PCR in one procedure, as there is no *E. coli* present to contaminate the results.

Based on time requirements, conjugation is more time consuming with one experiment spanning 3 days and taking a minimum of 6 hours if all tools are prepared. Electroporation experiments span 2 days and take about 5 hours if preparing the DNA is taken into account. If linearized DNA is available, the total time requirement for electroporation is less than 2 hours.

The obvious advantage of conjugation-mediated DNA transfer, based on the results in Figure 11.3, is the higher achieved efficiency. However if efficiency is not an important parameter, but generating few clones is enough, electroporation can be chosen over conjugation. Larger DNA fragments have been successfully transferred via conjugation in previous studies [Karas et al., 2015], which makes this method of genetic transfer preferable for larger constructs.

12.6 Future work

Several additional studies for the further optimization of the conjugation protocol can still be made. As mentioned previously, optimization of the recipient to donor ratio and overall concentration of recipient to donor needs to be investigated further. In particular, an additional increase in ratio and/or concentration for donor strain, as one of the highest conjugation efficiencies obtained was achieved with a ratio of 1:6 (pAPA0602-YST, Table 10.1).

In addition, shorter incubation times after the crucial 90 minute incubation can be tested, such as 1, 2, 4 and 6 hours. If a shorter time is found to yield good results, this can cut down the overall time of the protocol. The time may again be cut, if a lower OD_{600} of harvested bacterial donor cultures can be used without compromising the results.

Confirmation of transconjugants by colony PCR is not straightforward due to the contaminating presence of *E. coli* DNA around the transconjugants. Optimization to enable an easier colony PCR procedure is desirable if conjugation is to be used as a method of DNA transfer. This is important if conjugation is to be used a method in molecular biology on the same level as other transformation methods in *N. oceanica*.

It can also be further studied whether conjugation is actually temperature sensitive. This can elucidate more about the process and under which conditions it occurs.

Another Mu-free donor strain, MFDpir, could be tested to compare with DH10B/pTA-Mob and S17-1 [Ferrieres et al., 2010]. The advantage of MFDpir over DH10B/pTA-Mob would be the easier creation and maintenance of the donor strain (by heat shock transformation, one selection marker).

For growth optimization of Nannochloropsis oceanica, it would be interesting to investigate the effect of growing the cells in full salinity (instead of 50%) on the transformation efficiency of electroporation.

13 Conclusions

We know that conjugation-mediated transfer of DNA was successful in the heterokonts *P. tricornutum* and *T. pseudonana*. it can be transferred. This study concludes that transkingdom conjugation can be used as a method of DNA transfer in *N. oceanica* as well, using the successful protocol developed in this project.

Plasmid transfer mediated by *E. coli* DH10B/pTA-Mob resulted in the highest conjugation efficiency, between the two donor strains. This supports the use of DH10B/pTA-Mob over *E. coli* S17-1, in addition to previously reported advantages.

It was revealed that co-incubation of donor and recipient cells at 30 °C is required for establishing conjugation. Optimal results were achieved at 90 minutes. Optimization of the protocol revealed that the relationship between the ratio of donor to recipient cells and the efficiency of conjugation still need to be investigated. Further research also needs to be done before a conclusion can be reached about the role of the yeast sequence, *CEN6-ARSH4-HIS3* in mediating more efficient transformation of *N. oceanica*.

High transformation and conjguatoin efficiencies in *N. oceanica* were achieved using the pAPA0602 and pAPA0169, contrary to what has been reported previously for the plasmids. It was also revealed that the promoters nanno-602 and nanno-169 confer on to *N. oceanica* a similar level of tolerance to zeocin. Therefore, it can be concluded that these plasmids are useful tools for efficient transformant and transconjugant selection in *N. oceanica*.

A Growth experiment

Supplementary information from growth optimzation experiment. Table A.1 shows the composition of Guillard's f/2 medium.

Table A.1: Composition of f/2 medium and concentration of components per litre of filtered sea water.

Component	**Concetration**
$NaNO_3$	0.075 g/L
NaH_2PO_4	0.024 g/L
$CuSO_4$ 5H2O	0.98 mg/L
Na_2MoO_4 2H2O	0.63 mg/L
$ZnSO_4$ 7H2O	2.2 mg/L
$CoCl_2$ 6H2O	1.0 mg/L
$MnCl_2$ $4H_2O$	18.0 mg/L
Biotin	0.1 mg/L
Cyanocobalamin	1.0 mg/L
Thiamine	0.2 g/L

B Molecular cloning

Supplementary information for molecular cloning of pAPA0602, pAPA0169 and pSELECT100 plasmids.

B.1 Competent cells: media and transformation efficiency calculation

The media used during making of competent cells are described in Table B.2. Raw data and transformation efficiencies for competent cells is presented in Table B.1.

Calculation example of transfromation efficiency:

100 µL of 2 mL of cells were plated, so 1/20 of the cells were plated, therefore, 1/20 of the DNA is present.
There were 114 colonies counted on the plate.
0.05 x 0.01 = 5 x10^{-4}
165,6 ng/µL plasmid DNA x 5 x10^{-4} = 0,0828 ng DNA
From nanogram to microgram: 0.0828 ng /1000 = 8.28x10^{-5} µg
114 colony forming units (cfu) / 8.28x10^{-5} µg DNA
= 4.1 x 10^{6} cfu/µg DNA

Table B.1: Transformation efficiency of heat shock competent DH5α cells transformed with pUC19.

Plate	**Dilution**	**cfu**	**Plasmid DNA (ng/uL)**	**DNA (ug)**	**cfu/ug DNA**
Negative cont.	0,05	0	0	0	-
1	0,05	uncountable	165,6	828	-
2	5	uncountable	165,6	$8,28x10^{-4}$	-
3	5	114	165,6	$8,28x10^{-5}$	$4,1x10^{6}$
4	$5x10^{-5}$	16	165,6	$8,28x10^{-6}$	$1,9x10^{6}$
5	$5x10^{-6}$	9	165,6	$8,28x10^{-}7$	$1,0x10^{7}$
6	$5x10^{-7}$	0	165,6	$8,28x10^{-}8$	-
7	$5x10^{-8}$	0	165,6	$8,28x10^{-}9$	-

Table B.2: Components of SOC, TfBI, TfBII, and yB media are described in (a), (b), (c), and (d) respectively.

(a) SOC media (last three are filter sterilized and added after autoclavation)

Component	**Amount per litre**
Bactotryptone	20 g
Yeast extract	5 g
NaCl	0.584 g
KCl	0.186 g
$MgCl_2$	10 mL
$MgSO_4$	10 mL
1M glucose	20 mL

(b) TfBI media (filter sterilize, Adjust pH to 5.8 with CH_3COOH)

Component	**Amount per litre**
CH3CO2K	2.94 g
MnCl2	9.9 g
RbCl	12.1 g
CaCl2	1.48 g
Glycerol	150 mL

(c) TfBII media (pH adjustment with KOH)

Component	**Amount per litre**
100 mM MOPS pH 7	10 mL
$CaCl_2$	1.10 g
RbCl	0.12 g
Glycerol	15 mL

(d) yB media(Adjust pH to 7.6 with KOH, autoclave then add MgSO4)

Component	**Amount per litre**
Bactotryptone	20 g
Yeast extract	5 g
KCl	0.76 g
MgSO4	34 mL

B.2 Primers for Gibson assembly

Primers shown in Table B.3 were used to amplify the pAPA1001 plasmids' (backbone) to create fragments with overhangs suitable for Gibson assembly. Insert (Ins-) primers were used for amplification of *CEN6-ARSH4-HIS3* (insert) sequence and to create complementary overhangs to those in the backbone.

Table B.3: Gibson assembly primers for pAPA1001 backbones and *CEN6-ARSH4-HIS3* insert.

	Backbone
pAPA-fwd	5'-CCAAAGGTGTTCTTATGTAGAGATTATCAAAAAGGATCTTCACC-3'
pAPA-rev	5'-TTATTTTTATAGCACGTGATCATGACCAAAATCCCTTAACG-3'
	Insert
Ins-fwd	5'-GTTAAGGGATTTTGGTCATGATCACGTGCTATAAAAATAATTATAATTTAAA-3'
Ins-rev	5'-AAGATCCTTTTTGATAATCTCTACATAAGAACACCTTTGGTG-3'

B.3 Sequences

TAAGGCTCTTCNGCTAGACACAACGTGGCTTTCCCCCCCCCCCCTGCAGGTCGACGGATCTTTTC
CGCTGCATAACCCTGCTTCGGGGTCATTATAGCGATTTTTTCGGTATATCCATCCTTTTTCGCAC
GATATACAGGATTTTGCCAAAGGGTTCGTGTAGACTTTCCTTGGTGTATCCAACGGCGTCAGCCG
GGCAGGATAGGTGAAGTAGGCCCACCCGCGAGCGGGTGTTCC
TTCTTCACTGTCCCTTATTCGCACCTGGCGGTGCTCAACGGGAATCCTGCTCTGCGAG
GCTGGCCG
ATAAGCTCTAAGAAACCATTATTATCATGACATTAACCTATAAAAATAGGCGTATCACGAGGCCC
TTTCGTCTTCAAGAATTAATTCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGC
GTTACCCAACTTAATCGCCTTGCAGCACATCCCCCTTTCGCTNGAAGAGCAGAT

Figure B.1: Sequence including *oriT* (highlighted).

ATCACGTGCTATAAAAATAATTATAATTTAAATTTTTTAATATAAATATATAAATT
AAAAATAGAAAGTAAAAAAAGAAATTAAAGAAAAAATAGTTTTTGTTTTCCGAAGA
TGTAAAAGACTCTAGGGGGATCGCCAACAAATACTACCTTTTATCTTGCTCTTCCT
GCTCTCAGGTATTAATGCCGAATTGTTTCATCTTGTCTGTGTAGAAGACCACACAC
GAAAATCCTGTGATTTTACATTTTACTTATCGTTAATCGAATGTATATCTATTTAA
TCTGCTTTTCTTGTCTAATAAATATATATGTAAAGTACGCTTTTTGTTGAAATTTT
TTAAACCTTTGTTTATTTTTTTTTCTTCATTCCGTAACTCTTCTACCTTCTTTATT
TACTTTCTAAAATCCAAATACAAAACATAAAAATAAATAAACACAGAGTAAATTCC
CAAATTATTCCATCATTAAAAGATACGAGGCGCGTGTAAGTTACAGGCAAGCGATC
CTAGTACACTCTATATTTTTTTATGCCTCGGTAATGATTTTCATTTTTTTTTTTCC
ACCTAGCGGATGACTCTTTTTTTTTCTTAGCGATTGGCATTATCACATAATGAATT
ATACATTATATAAAGTAATGTGATTTCTTCGAAGAATATACTAAAAAATGAGCAGG
CAAGATAAACGAAGGCAAAGATGACAGAGCAGAAAGCCCTAGTAAAGCGTATTACA
AATGAAACCAAGATTCAGATTGCGATCTCTTTAAAGGGTGGTCCCCTAGCGATAGA
GCACTCGATCTTCCCAGAAAAAGAGGCAGAAGCAGTAGCAGAACAGGCCACACAAT
CGCAAGTGATTAACGTCCACACAGGTATAGGGTTTCTGGACCATATGATACATGCT
CTGGCCAAGCATTCCGGCTGGTCGCTAATCGTTGAGTGCATTGGTGACTTACACAT
AGACGACCATCACACCACTGAAGACTGCGGGATTGCTCTCGGTCAAGCTTTTAAAG
AGGCCCTACTGGCGCGTGGAGTAAAAAGGTTTGGATCAGGATTTGCGCCTTTGGAT
GAGGCACTTTCCAGAGCGGTGGTAGATCTTTCGAACAGGCCGTACGCAGTTGTCGA
ACTTGGTTTGCAAAGGGAGAAAGTAGGAGATCTCTCTTGCGAGATGATCCCGCATT
TTCTTGAAAGCTTTGCAGAGGCTAGCAGAATTACCCTCCACGTTGATTGTCTGCGA
GGCAAGAATGATCATCACCGTAGTGAGAGTGCGTTCAAGGCTCTTGCGGTTGCCAT
AAGAGAAGCCACCTCGCCCAATGGTACCAACGATGTTCCCTCCACCAAAGGTGTTCTTATGTAG

Figure B.2: The *CEN6-ARSH4-HIS3* sequence (insert of Gibson assembly), which was amplified from the pTpPUC3 plasmid.

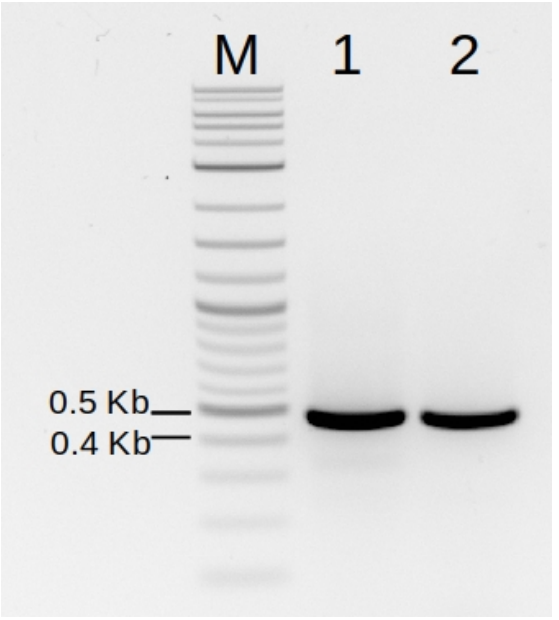
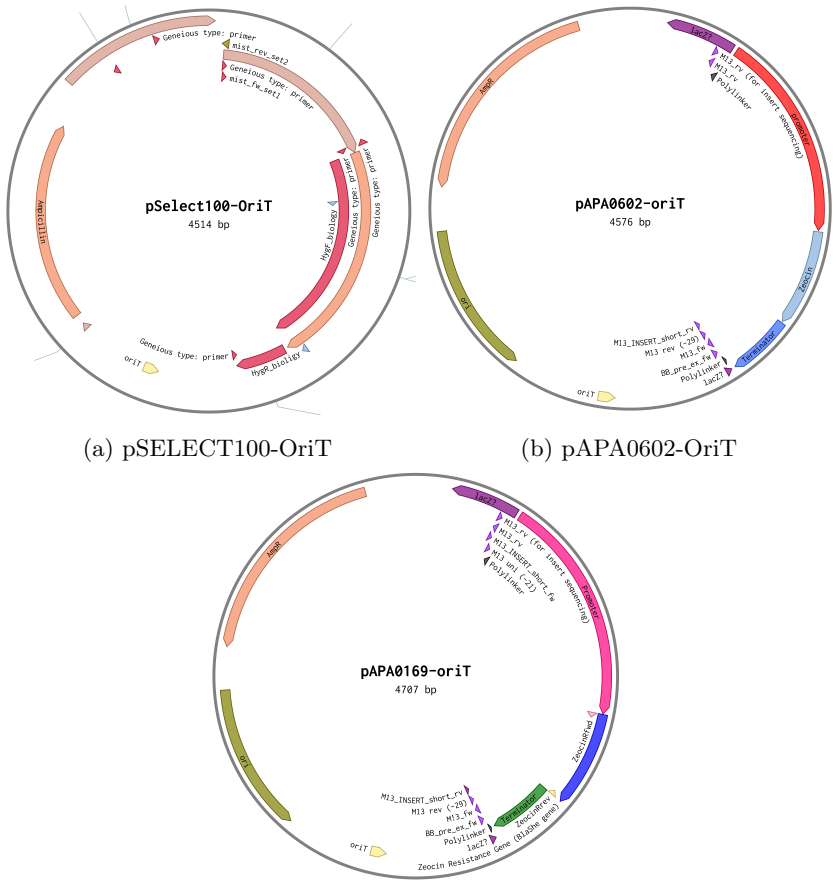


Figure B.3: PCR product of oriT amplification. The amplified sequences containing oriT matched the expected sizes of 481 bp (lane 1) and 487 bp (lane 2). M: quick Load Purple 2-Log DNA Ladder (0.1-10.0 kb), 1: oriT-KpnI PCR product (481 bp), 2: oriT-SapI PCR product (487 bp).

B.4 Plasmid maps

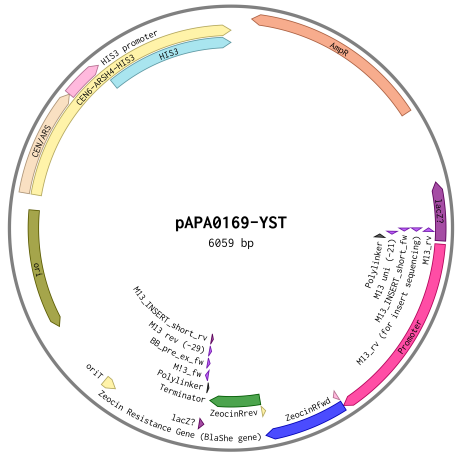


(a) pSELECT100-OriT

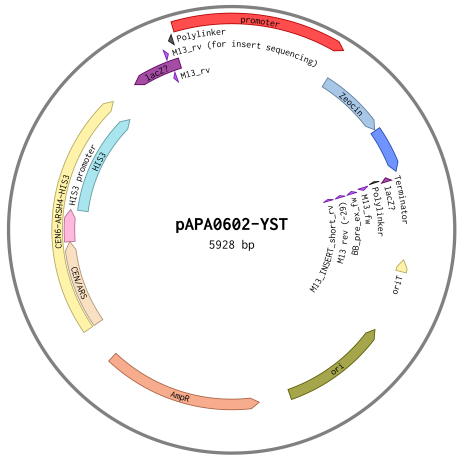
(b) pAPA0602-OriT

(c) pAPA0169-oriT

Figure B.4: Plasmids maps after the insertion of oriT.



(a) pAPA0169-YST



(b) pAPA0602-YST

Figure B.5: Plasmid maps after insertion of *CEN6-ARSH4-HIS3* sequence

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