Genetic Transformation of *Acutodesmus obliquus* and *Neochloris oleoabundans* by bacterial conjugation

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Abstract

Microalgae are photosynthetic organisms with a vast array of industrial applications, from the production of feed and food ingredients such as lipids and proteins, the synthesis of biofuels, and production of antibodies. A cost-effective production of microalgae-derived products is in many cases not achieved due to yet too low product yields and productivity, and inefficient or costly biorefinery of microalgae. Strain development is therefore required, but has been severely hampered by the lack of optimized transformation techniques for microalgae. One of the novel methods to do so is by bacterial conjugation, a process through which bacterial donor cells are capable of transferring DNA into a recipient cell. It is known to occur abundantly between bacteria, though has recently been implemented for transformation of diatoms as well. In this study the green microalgae *Acutodesmus obliquus* and *Neochloris oleoabundans* were successfully transformed using bacterial conjugation using *E. coli* as a donor, with the episomal pPtPuc3 plasmid, while genetic transformation with the episomal pTpPuc3 plasmid still requires confirmation. Protocols for the genetic transformation of both microalgae species were developed, but still require further optimization. The focus of the protocols' optimization efforts was conjugation time, concentration of zeocin or nourseothricin in the medium for transconjugant selection, and the period of culture exposure to light. For *A. obliquus*, a correlation between longer conjugation times and a higher number of colonies was found. Comparatively however, a 60-minute conjugation time showed a better colony ratio between transformants and control, while longer conjugation of 90 and 120 minute periods showed a small margin between the number of possible transformants and the number of colonies in the controls. Much like *A. obliquus*, the transformation of *N. oleoabundans* shows a relation between longer conjugation time and number of colonies, with a two-hour period generating a greater number of colonies. For *N.oleoabundans* no link was found however between shorter incubation periods and advantageous non-confirmed transformant/control colony ratio. In addition, it was found the number of colonies was increased by prolonging the cultures' exposure to light. This is the first reported instance of green microalgae being genetically transformed by bacterial conjugation, and only the second transformation method used successfully on *A. obliquus* and *N. oleoabundans*.

Keywords: *Neochloris oleoabundans*, *Acutodesmus obliquus*, Green microalgae, Conjugation, Genetic transformation

Introduction

Microalgae microscopic photosynthetic are eukaryotic organisms. Though most are unicellular, several described species are colony forming, namely species of the *Volvox* genus and *Botryococcus braunii*. Microalgae have seen a resurgence in biotechnological research in the last decades, a lot of which focused towards the production of compounds of interest to a vast range of industries [1], offering an alternative to conventional methods of production, namely agriculture. Amongst the most sought after applications resulting from microalgae cultivation it is possible to name the production of bioactive products, used in the pharmacological and cosmetic industries, lipids for the production feed, food or biofuels such as biodiesel and treatment of industrial and municipal wastewater mainly through the removal of phosphates, nitrates and heavy metals [1].

The improvement of microalgae has been recognized as an essential requirement to make low-value compound production economically sustainable, as none of the microalgae species characterized so far have the necessary traits to make them economically suitable in an industrial setting. Though genetic optimization of algal strains has received little attention [2] and few species have been the focus of this kind of research [3], it has been theorized that several aspects of microalgae have the potential to be improved through genetic modification. The main challenge regarding the genetic transformation of microalgae resides in the fact that due to their immense diversity no single transformation method or protocol can be used for all species, meaning that a thorough study of each organism is needed before an optimal transformation toolkit is devised for a given alga [3]. Conventional methods to transfer DNA into microalgae include electroporation, microparticle bombardment, *Agrobacterium tumefaciens*-mediated transformation (ATMT) and the glass bead transformation method.

Conjugation is a DNA transfer process from one cell to another, requiring physical contact between donor and recipient cells [4]. Though conjugation is more closely associated with the DNA transfer between bacteria, it is possible that any type of cell can serve as a recipient since all events that occur during conjugation are wholly dependent on the donor cell and much of the necessary machinery is generally plasmid-encoded [4]. Conjugation has yet to be shown to be an appropriate method for the genetic manipulation of microalgae, however it has been successfully employed to genetically modify other eukaryotic organisms like yeast [5], mammalian [4] and diatom cells [6]. Karas *et al.* (2015) [6] reported being able to transformation the diatoms *T. pseudonana* and *P. tricornutum* using bacterial conjugation. Given the phylogenetic proximity of diatoms to green microalgae it would seem feasible to use the same approach for these same organisms.

Materials and Methods

Organisms and growth conditions Stock cultures of *Neochloris oleoabundans* UTEX 1185 and *Acutodesmus obliquus* SAG 276-6 were grown in fresh water (FW) solid media. All organisms were maintained under a repeating 16:8 light/dark cycle (L/D cycle) at a light intensity of 50-60 µmol m^{-2} s^{-1} and 25°C. Liquid cultures of both green microalgae were grown and maintained under repeated 16:8

L/D cycle at a light intensity of 50-60 µmol m^{-2} s^{-1}, 25°C and 100 rpm.

Stock cultures of *Escherichia coli* DH10B containing the cargo plasmids pPtPuc3 and pTpPuc3, were grown overnight in LB media complemented with kanamycin (50 and 100 µg/ml) at 37°C, after which the cultures were maintained at 5°C. Both *E. coli* strains also contained the conjugative plasmid pTA-Mob, necessary for the cargo plasmids to be transferred.

Growth monitoring Growth of green algae liquid cultures was monitored by measuring optical density at 750 nm (OD_{750}) using a spectrophotometer (Hach Langer DR-600) and measuring cell concentration (X) using a Coulter Counter (Beckman Coulter Multisizer™ 3). The optical density of *N. oleoabundans* and *A. obliquus* samples was measured using FW medium as blank solution. For monitoring of X, *N. oleoabundans* and *A. obliquus* samples were prepared using an adequate amount of Isoton buffer (Coulter® Isoton® II diluent), for a total volume of 20 ml.

Development and optimization of a bacterial-mediated conjugation protocol for *A. obliquus* and *N. oleoabundans* The procedure to transform the green microalgae *A. obliquus* and *N. oleoabundans* through bacterial conjugation followed the same work-flow used to produce *P. tricornutum* transconjugants by Karas *et al.* (2015), with the necessary modifications to the protocol in order to favour the growth of the green microalgae.

From an exponentially grown microalgae culture, 250 µl (adjusted to 5.5 x 10^7 cells/ml for *A. obliquus* and 1.0 x 10^8 cell/ml for *N. oleoabundans*) forward plated on 1.6% agar FW (Fresh Water) medium plates and incubated at 25°C, under a 16:8 L/D cycle (50-60 µmol m^{-2} s^{-1}), for 6 to 10 days. After the incubation stage, the cells were scraped from the plates with 500 µl of FW media, collected, counted and the concentration of the cell suspension adjusted to 2 x 10^8 cells/ml for *A. obliquus* and 5 x 10^8 cells/ml for *N. oleoabundans*.

Both *E. coli* strains were grown in 50 ml LB cultures overnight (37°C, 150 rpm) and their optical density at 600 nm (OD_{600}) adjusted to 0.8 – 1.0. These cultures were then spun down in 50 ml sterile tubes (10 minutes at 3000 x *g*) and the resulting pellets were resuspended in 500 µl of SOC medium.

For the transfer of DNA, 200 µl of green microalgae and *E. coli* culture were added to 1.5 ml epperdorf tubes and mixed by pipetting up and down. The mixtures were plated on 1.6% FW agar plates with 5% LB and incubated in the dark, at 30°C, for three different conjugation time periods: 60, 90 and 120 minutes. This was followed by recovery incubation at 25°C, under 16:8 L/D cycle (50-60 µmol m^{-2} s^{-1}) for 2 days.

Following the recovery period, 1 ml of FW medium was added to the plates for the cells to be scraped, and 200 µl of resuspended cells were plated on FW selection plates supplemented with different concentrations of zeocin or nourseothricin; and 1 µg/ml of gentamycin, for the transconjugant selection. These cultures were maintained at 25°C and 20-30 µmol m^{-2} s^{-1} until isolated colonies were observed.

Once isolated colonies of sufficient size were observed, several were picked and subsequently replated on solid FW medium supplemented with the same concentration of antibiotic. These cultures were then maintained at 25°C and 20-30 µmol m^{-2} s^{-1}, until enough biomass

was obtained for both microalgae DNA extraction and for part of the colonies to be replated onto LB plates to verify the possible presence of bacteria in the media.

Total DNA extraction Total DNA extraction of selected microalgae and bacterial colonies was conducted to confirm the presence of the transferred plasmid molecule. The biomass from the selected colonies was dissolved in 300 µl of SDS in bead beater tubes. Immediately after, 150 µl of vortexed Chloroform: Phenol: Isoamyl alcohol (25:24:1) solution was added to the tubes. The collected cells were then lysed using a bead beater (Bertin, Precellys 24) at 4000 rpm and 2x 60s periods (with 20 seconds between them). After lysis, the samples were spun down for 5 min at 13400 rpm.

Following cell lysis, 200 µl from the top phase of the samples was recovered (without touching the beads), and added to a 1.5 ml Eppendorf, as well as 20 µl of sodium acetate (1/10th of total volume of the collected sample). This was followed by the addition of 400 µl of cold ethanol (100%) and placing the tubes in the cold (-23ºC) for 1 hour. Subsequently the samples were centrifuged for 15 min at 14000 rpm and 4ºC. The supernatant was carefully removed without disturbing the pellet and 250 µl of cold ethanol (70%) was added. The samples were spun down one final time for 5 min, 14000 rpm and 4ºC. The entirety of the supernatant was discarded and the remaining ethanol was removed by placing the open tubes at 37ºC.

The extracted DNA was resuspended by adding 15 µl of water (RNase and DNase free) and pipetting up and down several times and a quick spin down to precipitate all of the extracted DNA to the bottom of the tubes was performed. The DNA was quantified using a Nanodrop reader. After the extraction of DNA from the chosen algal and bacterial colonies, the concentrations of the same were homogenised in order to obtain comparable results.

Colony PCR confirmation of transformants Polymerase Chain Reaction (PCR) was used to confirm the transconjugants resulting from conjugation from *E. coli* to the green algae. The confirmation was based on the amplification of a fragment of the zeocin resistance marker (*shble* gene) using primers Forward-Zeo (5'- ATG GCC AAG TTG ACC AGT GC -3') and Reverse-Zeo (5'-TCA GTC CTG CTC CTC GGC -3'). In addition to the samples, a blank (containing all of the PCR reagents with the exception of the DNA template), a negative control (C-, DNA extracted from colonies replicated from control plates or wild-type strain) and a positive control (C+, pPtPuc3) were also tested for the presence of the *shble* gene. The PCR reactions were run with an initial denaturation for 5 minutes at 95ºC and 40 cycles of 30 seconds at 95ºC, an annealing step for 30 seconds at 59ºC for the *sh ble* gene sequence and extension for 80 seconds at 72ºC. A final extension step, took place for 5 minutes at 72ºC. The PCR products were then indefinitely kept at 4ºC. The PCR products were separated by agarose electrophoresis gel at 80 V, using GelRed in order to stain the amplified DNA.

Results and Discussion

***N. oleoabundans* transformation through bacterial conjugation** The first transformation attempt of *N. oleoabundans*, using the episomal pPtPuc3 plasmid, was perfomed using zeocin

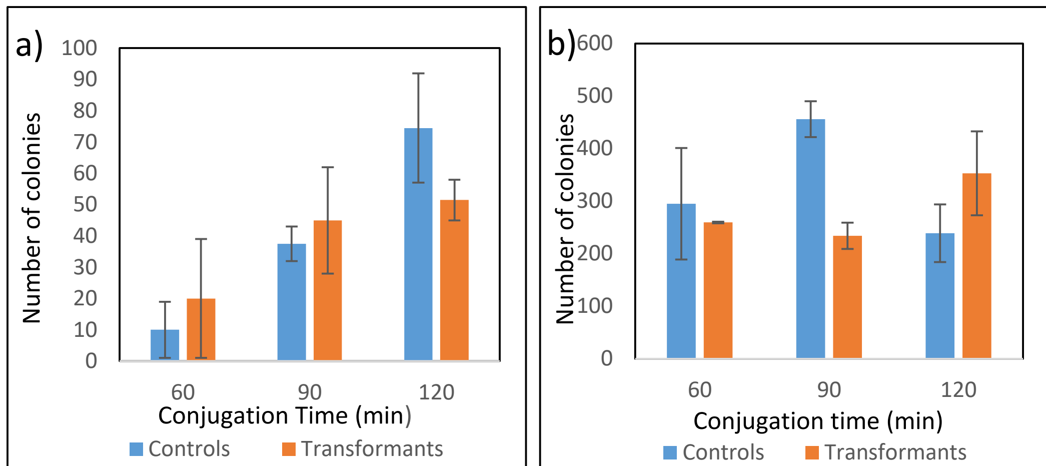

Figure 1a: Number of *N. oleoabundans* colonies, after 14 days of growth, in the selection plates supplemented with 20 µg/ml of nouseothricin, different conjugation periods and under a 16:8 L/D cycle. **Figure 1b:** Number of *N. oleoabundans* colonies in the selection plates supplemented with 20 µg/ml of nouseothricin, and different conjugation periods under a 24:0 L/D cycle. Error bars denote the standard deviation of the mean from two biological replicates per condition.

concentrations of 35 and 40 µg/ml in the media for selection of transconjugants. The low number of colonies present in the selection plates combined with standard deviations reaching up to 100%, along with the time period necessary for them to become visible (25 days), indicate that the zeocin concentrations used were far beyond the natural resistance of *N. oleoabundans,* as well as the higher resistance threshold provided by the zeocin resistance gene present in pPtPuc3. This meant that no statistically relevant information on the efficiency of conjugation as a method of microalgal transformation, or the effect of the different conditions, were obtained.

The transformation of *N. oleoabundans* using bacterial mediated conjugation was attempted in two more trials, both using 20 µg/ml of nourseothricin for the selection of transformants and L/D cycles of 16:8 and 24:0 (figure 1). From the data we can distinguish a pattern: the longer the conjugation time, the greater the average number of colonies formed, where in both cases the longer conjugation period (120 minutes) produced the highest average number of colonies. An exception to this trend would be the 90-minute conjugation assay in figure 1b, where the number is lower than that of 60-minute incubation. While in figure 1a there is a steady increase in the average number of colonies as the conjugation period increases, the same is not observed when the plates were exposed to constant illumination (figure 1b).

In these trials, the number of colonies present in the control plates was expected to be lower than their corresponding transformant plates. However, that was not the case in the control plates of 120-minute conjugation assays (figure 1a) as well those assigned 60 and 90 minutes of conjugation in the third trial (figure 1b). Considering that the colonies in these controls cannot possibly be composed of resistant cells it is more likely that the reasons for these disparities are inactivation of the antibiotic due to the long incubation period.

There is one more important piece of information to be extracted from these last two assays: a longer exposure to light, with the same light intensity, favours the survival of more cells, as

evidenced by the higher number of colonies counted in these conditions (figure 1b). Though it is possible that the longer exposure to light, could stimulate greater cell multiplication and growth, there is also the chance that this same factor could result in a higher deactivation rate of the antibiotic present in the media, in turn reducing the selective pressure meant to eliminate false positives. To explore these scenarios, it would be necessary to verify the presence of the pTpPuc3 plasmid in the possible transformant colonies through the use of PCR.

Because both *A. obliquus* and *N. oleoabundans* are known to form associations with several other bacterial species [7], it also became necessary to replate part of the algal biomass onto LB plates to collect DNA from bacteria present in the FW media. As such there could always be the possibility that *E. coli* could serve as a DNA donor to more than one organism in the same culture (even if the number of microalgal cells should far outnumber bacteria). It would also be conceivable that *E. coli* cells could still be present in the media as the growth conditions could allow for the growth of this bacterium in spite of the lack of carbon source in the medium (though this could be obtained from dead cells). Replating several colonies onto LB plates revealed that there were indeed bacteria present in association with the microalgae, but since no bacterial colonies could be seen in the FW selection media, most of the extracted DNA from the latter media should likely be of microalgal origin.

PCR reactions were performed to amplify the *shble* gene sequence, which had an expected band size of approximately 370 bp. Figure 2 shows

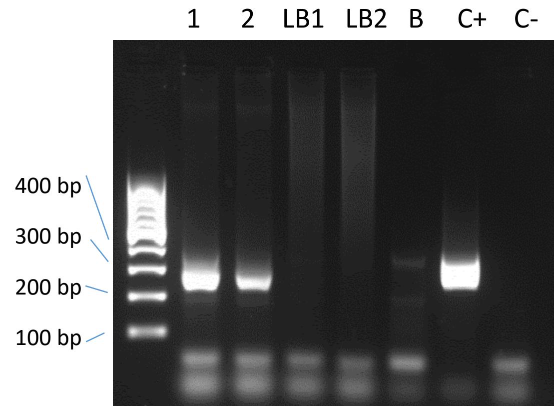

Figure 2: Amplification of the zeocin resistance gene (*sh ble*) present in the pPtPuc3 plasmid, after DNA extraction from *N. oleoabundans* and bacterial colonies. **Legend:** (1,2) *N. oleoabundans* DNA samples, (LB1, LB2) Bacterial DNA samples, (B) blank, (C+) positive control, (C-) negative control (wild-type strain).

both the results for the amplification of *shble* sequence from *N. oleoabundans* algal colonies present in FW media (lanes 1 and 2) and the corresponding bacterial colonies found in LB plates (lanes LB1 and LB2). While the number of potentially transformant colonies to choose from, containing the pPtPuc3 plasmid, was extremely limited the few colonies that could be found were replated onto FW media with the same zeocin concentration, so that enough biomass could be collected for DNA extraction for confirmation that conjugation took place, and that false positives could be eliminated. Once colonies had grown sufficiently their DNA was extracted and used as templates for amplification of the *shble* gene (responsible for the zeocin resistance), only present in the pPtPuc3 plasmid. The same was carried out for the extracted bacterial DNA.

Both DNA samples from *N. oleoabundans* colonies showed a strong bands of comparable size to that of the positive control (pPtPuc3 plasmid), while neither of the corresponding bacterial

samples showed any such DNA sequences. Two weak bands were found in the blank sample; however, neither was of the same size as that of the positive control or any of the other amplified DNA sequences, possibly as consequence of contamination. All the sequences diverged slightly from the expected size as they are smaller by approximately 120 bp. Since this also seems to have affected the amplified fragment in the positive control it would be feasible that these inconsistences could have been caused by heterogeneity of the electrophoresis gel or due to the interaction with Gelred [8]. As an alternative to Gelred, using a post-staining method rather than pre-staining could potentially correct these size inconsistences [8]. The presence of amplified DNA sequences in the *N. oleoabundans* DNA samples of the same size as the positive control, in conjunction with the absence of the same from the bacterial samples, confirms that microalgae transformants were successfully produced.

***A. obliquus* transformation through bacterial-mediated conjugation** The first genetic transformation assay of *A. obliquus* through conjugation, was tested using the same concentration of zeocin (35 and 40 µg/ml) as *N. oleoabundans* and three different times of conjugation (60, 90, 120 minutes) (figure 3). In figure 3a we can see that as the time of conjugation increases, so does the number of isolated colonies observed in growth medium, with 120 minutes leading to the highest number of colonies, while allowing conjugation to occur for 60 minutes produces a much lower number of colonies. Due to the high amount of residual growth present in the plates it was not possible to properly count the number of colonies. As a result, all trials assigned to

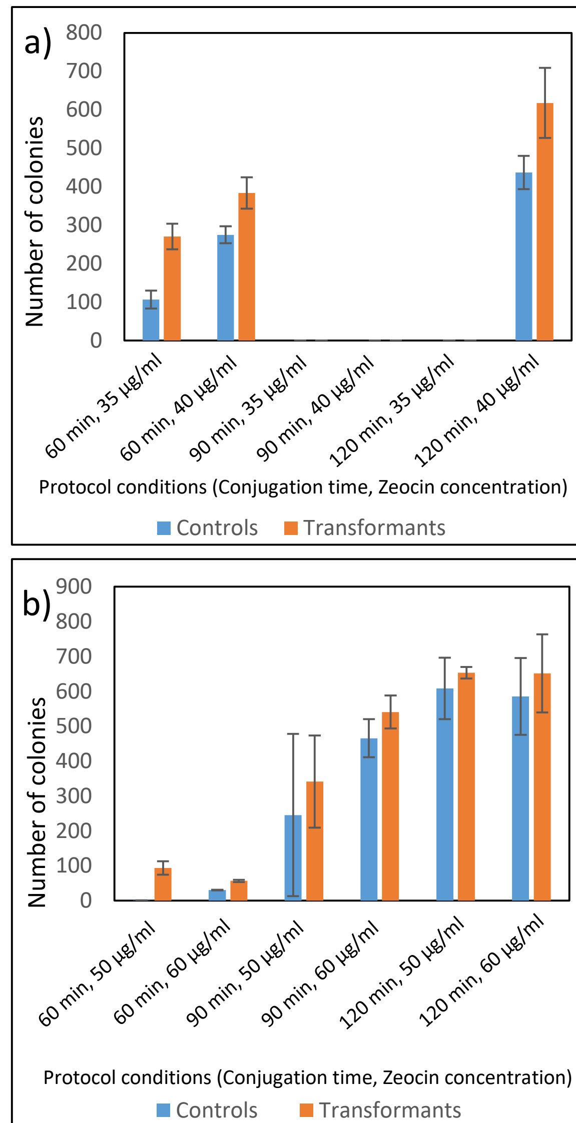

Figure 3a: First trial of *A. obliquus* transformation through conjugation. Number of *A. obliquus* colonies in the selection plates supplemented with different concentrations of zeocin, and different conjugation periods. **Figure 3b:** Second trial of *A. obliquus* transformation through conjugation. Number of *A. obliquus* colonies in the selection plates supplemented with different concentrations of zeocin, and different conjugations periods. Error bars denote the standard deviation of the mean from two biological replicates per condition.

90 minutes of conjugation and one assigned to 120 minutes of conjugation, no colonies could be identified.

To overcome this issue zeocin concentration was increased from 30 and 35 µg/ml, to 50 and 60 µg/ml, which led to much clearer colonies on plates. The results are compiled in figure 3b. Like in figure 3a, when conjugation is prolonged from 60 to 120 minutes, a 6-fold increase in colony forming cells can be seen. Comparing figures 3a to 3b, it was observed that the increase in concentration of zeocin in the medium significantly decreased the number of colonies in the plates incubated for 60 minutes. This is in line with an increase in antibiotic concentration. On the other hand, the number of identified colonies in the plates incubated for 90 and 120 minutes using higher zeocin concentrations is greater. This might be that a large number of smaller colonies were still concealed by the background growth in trial 2 (figure 3a). In any case, 120 minutes of conjugation once again produces more colonies, followed by 90 minutes of conjugation than a 60-minute incubation, the same behaviour previously observed in the *N. oleoabundans* transformation trials.

While the longest conjugation period produces the highest number of possible transconjugants, it also leads to the presence of the highest number of non-resistant colonies in the controls. In contrast, 60-minute conjugation assays produced the lowest number of colonies in each of the trials, but it always showed the best transformant to control colony ratio in each of the *A. obliquus* transformation trials, as seen in figures 3 and 4. While unclear as to why this may be the case, the fact that the mixed cultures incubated at 30°C for greater periods of time may have stimulated bacterial growth. This could result in *E. coli* being present in the medium in greater concentration once part of the culture was

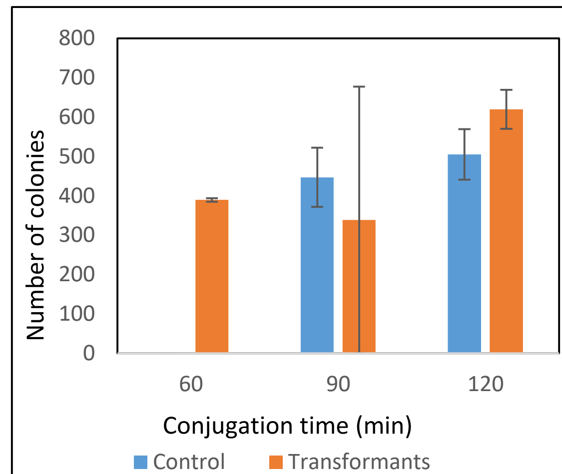

Figure 4: Number of *A. obliquus* colonies counted in selection plates supplemented with 40 µg/ml of nourseothricin, and different conjugation periods (60, 90 and 120 min) under a 16:8 L/D cycle. Error bars denote the standard deviation of the mean from two biological replicates per condition.

transferred to the selective media. Once in the selective medium, the greater number of cells in the culture could have reduced the selective effect of the antibiotic. This would account for the increase in colony number for both controls and transformant cultures. The increase in colony numbers as the antibiotic concentration increases (figure 3) however, could be a consequence of the decrease in background growth. The amount of background growth decreases with higher concentrations of zeocin, making colonies that would otherwise be hidden, visible. Based on these data, when developing a transformation protocol for *A. obliquus* it might prove more beneficial to use a shorter conjugation time as the chance of false positives is lower. Nevertheless, the fact remains that a confirmation of the presence of the plasmid in these would still have to be obtained.

Lastly, an attempt to transform *A. obliquus* using *E. coli* containing the pTpPuc3 was carried out, with the resulting transformants being selected on FW media supplemented with 40 µg/ml

of nourseothricin (figure 4). Much like in the last trials, the plates incubated for 120 minutes were found to have more colonies, on average. As a result of the complete growth inhibition in one of the 90-minute conjugation replicates, it wasn't possible to determine if the average number of colonies for this condition would be in relation to the other to conjugation periods. Unlike what had previously been seen, 60 minutes of conjugation showed a remarkably high number of isolated colonies when transformed with pTpPuc3, without the presence of a similar number of colonies in it respective controls, being the sole case in which there was no colonies found of the controls. Further proof that a 60-minute conjugation period could the a more beneficial condition for the transformation of *A. obliquus*.

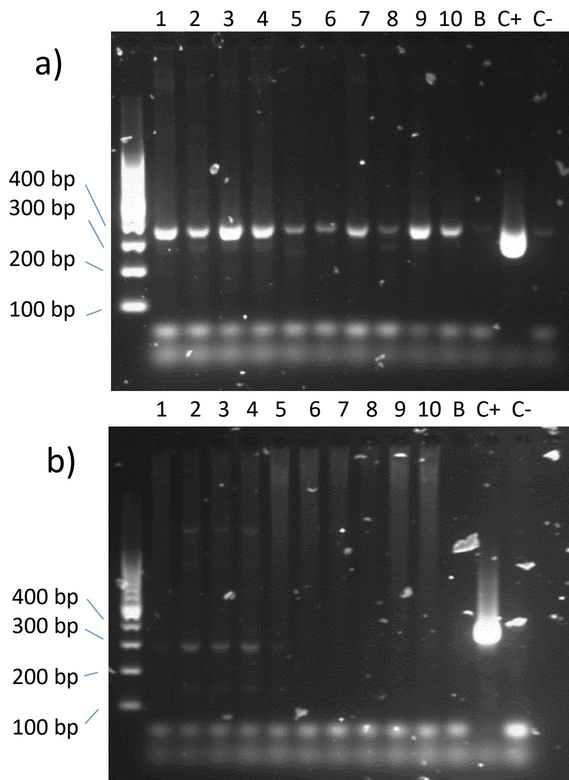

Figure 5a: Amplification of *the shble* gene sequence from DNA extracted from 10 random *A. obliquus* colonies. **Figure 5b:** Amplification of the *shble* gene sequence from DNA samples extracted from bacterial colonies found in the corresponding LB plates. **Legend:** (1-10) DNA samples, (B) blank, (C+) positive control, (C-) negative control (wild-type strain).

In order to confirm that conjugation had occurred PCR amplification for the *shble* gene was performed on both algal and bacterial DNA. The first PCR was meant to amplify the *shble* gene from microalgal colony DNA samples (present in the pPtPuc3 plasmid); and a second PCR where the *shble* gene would also be amplified from bacterial DNA samples collected from LB plates. Amplified DNA sequences of around 370 bp would serve as preliminary confirmation of successful transformation. The DNA samples from all ten microalgae samples showed the presence of an amplified sequence with comparable size to that of the positive control of about 370 bp (figure 5a). Figure 5b shows the separation of fragments amplified from bacterial DNA samples. In this case no fragments of the same size to that of the positive control were produced, though three slightly shorter bands, close to 300 bp, can be seen in three of the bacterial DNA samples. Unexpectedly, similar sized (but much weaker) bands can be seen for the blank and the negative control in figure 5a, which are absent in figure 5b.

It is uncertain whether the smaller weak bands in several of the DNA samples (close to 300 bp) in figure 5b were visible due to the presence of residual microalgae DNA or if some of the bacterial cells also contained the pPtPuc3 plasmid. It is worth noting however that unlike the bands seen in figure 5a, that none of the weak 300 bp bands seen in figure 5b have the same length as that of the positive control, and so it also be indicative that this a result of the amplification of non-specific sequences. The presence of faint fragments in blank

and negative control in figure 5a, should in all likelihood be explained by contamination of the samples as these same bands were not present in figure 5b.

Given the self-replicating and high copy number properties of the pPtPuc3 plasmid it seems likely that even without the presence of a selective pressure in the LB media, that a number of the cells could still possess the plasmid had they received it by conjugation from the *E. coli* donor cells (justifying the presence of weak amplified sequences). It stands to reason that the *A. obliquus* colonies from which the DNA was collected were in fact zeocin resistant transconjugants, as the corresponding bacterial DNA samples did not show any amplified DNA sequences of comparable intensity to that of the positive control or the bands obtained from the *A. obliquus* DNA samples.

Conclusion

This is the first reported case of green microalgae being genetically modified using bacterial conjugation, and the second reported transformation method for both *A. obliquus* and *N. oleoabundans*. While a complete optimization of the transformation protocol was not concluded, it was determined at for *N. oleoabundans* a conjugation period of 120 min produces the highest number of colonies. The same was seem for *A. obliquus,* however, a shorter conjugation time of 60 minutes appears to be best, as it reduced or even eliminates colonies from the controls which could suggest the presence of fewer false positives. Should the protocol be properly optimized is it possible that bacterial-mediated conjugation could be a more reliable transformation method for *A. obliquus* and *N. oleoabundans* than electroporation.

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