Chapter 1

Microalgae and Cyanobacteria as Green Molecular Factories: Tools and Perspectives

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Additional information is available at the end of the chapter

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Abstract

Cyanobacteria and eukaryotic microalgae are phototrophic microorganisms capable of producing organic compounds using solar energy. Owing to their fast growth, low cost cultivation, and the diversity of high-value chemical substances produced, they are considered an attractive target to be exploited by the biotechnology industry. While genetic modulation of these organisms has been extensively proved in the laboratory, present-day microalgal industry uses mainly non-transgenic strains. Although some unicellular cyanobacteria can be successfully engineered, many commercial bioproducts are synthesized preferably by eukaryotic microalgae or filamentous cyanobacteria to take advantage of their better-suited natural physiological characteristics. The successful genetic engineering of these microorganisms is not limited to the understanding of the gene expression machinery (e.g., promoters, codon usage, ribosome binding sites), but it must also include other subjects, such as defense mechanisms against the intrusion of foreign DNA. This chapter reviews current strategies in microalgae biotechnology and analyzes the most frequent problems we faced to genetically manipulate these microorganisms, including their transformation and selection methodologies. In summary, we attempt to provide a comprehensive review of the relevant information and tools required for optimal engineering of a photosynthetic microorganism employed in sustainable biotechnology applications.

Keywords: microalgae, cyanobacteria, transgenic strains, biotechnology, transformation

1. Introduction

Ancestors of present-day cyanobacteria developed the oxygenic photosynthesis as far back as 3.6 billion years ago [1], and the primary endosymbiotic event at the origin of all photosynthetic eukaryotes can be traced to 1.8 billion years ago [2]. It is estimated that algae are a group of about 72,500 species worldwide [3], with sizes varying from unicellular to giant multicellular organisms. On contrast, cyanobacteria is a monophyletic bacterial phylum composed by unicellular and multicellular (clusters, filaments, and ramified filaments) forms, including cellular differentiation in some filamentous representatives, and whose morphological complexities do not represent strictly an evolutionary history [4].

Eukaryotic microalgae and cyanobacteria are widely distributed, inhabiting aquatic (from marine to freshwater environments) and terrestrial ecosystems, and even extreme environments [5, 6]. They are responsible for ~50% of global primary production, fixing a substantial amount of carbon dioxide, especially in oligotrophic marine surfaces [5, 7]. In addition, diazotrophic cyanobacteria are major players in global nitrogen fixation [8], and many produce unique secondary metabolites, such as toxins [9].

These photosynthetic microorganisms have been cultivated for a long time as a food source and complementary diet for humans and also for the fish, shrimp, and poultry industries. Microalgae and cyanobacteria naturally produce diverse compounds of commercial interest such as proteins, carbohydrates, lipids, pigments, and alcohols; and some of their subproducts are currently commercialized by the pharmaceutical and cosmetic industries [10, 11]. Current industrial processes based on microalgal biotechnology mainly use non-transgenic strains. However, recent research has improved the genetic modulation in microalgal and cyanobacterial strains, despite the popular skepticism and mistrust surrounding the generation of transgenic strains, in particular, the potential generation of antibiotic resistant microorganisms. These advances have paved the way to obtain phenotypes that can considerably improve the production of high-valued compounds. The genetic transformation systems have been better developed in unicellular cyanobacteria because their amenability to genetic modulation. Nevertheless, progresses in the eukaryotic microalgae modification for the synthesis of several products have been achieved too, pushed by the idea of taking advantage of their natural phenotypic characteristics [12].

This chapter reviews current biotechnology strategies in microalgae and highlights the most frequent constraints for the genetic manipulation of these photosynthetic microorganisms. The emphasis of this analysis is focused on successful experiences that overcame previously described problems.

2. Microalgal and cyanobacterial biotechnology: from natural to transgenic strains

Considering the enormous diversity of microalgal and cyanobacterial species, they represent a unique reservoir of potentially novel commercial compounds. They have been used for the

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production of biomass and extraction of commercially attractive compounds for industries relative to human nutrition and health (e.g., nutraceuticals, sunscreens, pharmacological bioactives), agro- and aqua-culture (e.g., bio-fertilizers, food supplements), and renewable fuels (e.g., fatty acid methyl esters, alcohols, and hydrogen) [10, 11]. Eukaryotic microalgae are interesting due to their cellular storage of compounds (e.g., lipids, starch, and proteins), while cyanobacteria are relevant for the production of unique molecules (e.g., toxins) or molecules that can be secreted (e.g., ethanol, butanol, exopolysaccharides).

Unicellular cyanobacteria rarely produce large quantities of commercially attractive bioproducts. Thus, the genetic manipulation of them is normally oriented to increase the production of biofuels and other chemicals (e.g., [13, 14]). On the other hand, eukaryotic microalgae and filamentous cyanobacteria are more complicated for genetic modification. In the former, the foreign DNA needs high energy to trespass cell walls and membrane compartments; whereas the segregation of mutants is more difficult in multicellular organisms, such as filamentous cyanobacteria. Nonetheless, these organisms are good biotechnology prospects because they naturally accumulate commercially interesting compounds.

2.1. Biofuels

Over past decades, the energy industry has faced the challenge of providing new energy sources, and as new climate policies demand, it has to fulfill the requirement of finding ecologically friendly alternatives [15]. In this context, biofuel production from microalgae biomass is an alternative that captures and sequesters atmospheric carbon dioxide, the main greenhouse gas responsible for the current global warming [16].

In general, biofuels are classified into four main types: biodiesel, bioethanol, biogas, and biohydrogen. Below, we discuss examples of wild-type or genetically modified cyanobacterial and microalgal strains that produce biofuel [17, 18].

2.1.1. Biodiesel

Relative to their size, microalgae can store high amounts of lipids, especially intracellular oils, that can yield biodiesel through chemical (transesterification) and physical (distillation and cracking) conversions [16]. As the lipid composition differs among microalgae species, not all of them are useful for the production of biofuels. Some species of the *Nannochloropsis* genus are one of the better-suited microalgae for biofuel production. They are able to accumulate considerable amounts of intracellular lipids into vesicles (up to 70% of the cell dry weight), especially under stress conditions, and mainly in the form of triglycerides [19, 20]. Other examples of lipid-producing microalgae are *Chlamydomonas reinhardtii, Dunaliella salina, Botryococcus braunii*, and various *Chlorella* species [21, 22].

Genetic strategies used to generate strains of microalgae with greater ability to produce lipids include both knockout and overexpression of genes involved in lipid synthesis. These approaches have had diverse results. The overexpression of genes that belong to lipid synthesis pathway (ACCase, KASIII) increased the enzymatic activity, but did not increase the amount

of cellular lipids in different organisms [23]. Other approaches consistent on blocking metabolic pathways that promotes lipid synthesis. In this context, it has been reported that mutant strains of *C. reinhardtii*, where the ADP-glucose pyrophosphorylase or isoamylase genes (starch synthesis) had been disrupted, were able to accumulate higher amounts of lipids [24].

On the other hand, cyanobacteria accumulate lipids in thylakoid membranes at fast growth rate when high levels of photosynthesis are detected, reaching ~40% of dry biomass in unicellular cyanobacteria [25]. Genetic modifications of model cyanobacteria to overproduce, and even excrete, fatty acids have also been demonstrated [26–28]. However, redirecting the carbon metabolisms has detrimental effects, which limit the expected yield in some engineered strains [29]. Modifications of expression of genes related to mitigate the stress in *Synechococcus elongatus* PCC 7942 (e.g., overexpression of ROS-degrading proteins) resulted in the recovery of the fitness [27]. Otherwise, the selection of more resistant strain can be crucial for successful genetic modifications. For example, an engineered strain of *Synechococcus* sp. PCC 7002 yielded high levels of fatty acids without the detrimental effects observed in *S. elongatus* PCC 7942, when subjected to similar genetic modifications [28].

Furthermore, several prokaryotic and eukaryotic organisms naturally produce hydrocarbons (e.g., alkanes or alkenes), a direct fuel compatible with the existing fossil fuel infrastructure [30]. Cyanobacteria produce mostly C17 and/or C15 long-chain alkanes, using fatty acid precursors via two different pathways. One pathway involves two step enzymatic reactions driven by an acyl–acyl carrier protein reductase (AAR) and an aldehyde-deformylating oxygenase (ADO) [31]. The other known pathway produces alkenes through a multidomain protein homologous to type I polyketide synthases (PKS), which convert fatty acyl-ACP to α-olefin via elongation–decarboxylation mechanisms [32]. Molecular engineering attempts to enhance the production of alka(e)nes in unicellular cyanobacteria (e.g., *Synechocystis* sp. PCC6803 [33]), and in filamentous nitrogen-fixing cyanobacterium (*Anabaena* sp. PCC7120 [34]) of these two pathways generated low production of alka(e)nes.

2.1.2. Bioethanol

Like aliphatic hydrocarbon, alcohol-based biofuels can be used directly to power diesel engines. Ethanol is the most common alcohol-based biofuel synthesized via fermentation driven by heterotrophic microorganisms. Microalgae and macroalgae are good sources of carbohydrates (in the form of glucose, starch, and other polysaccharides) and proteins, which are used as raw material to produce bioethanol through various processes of hydrolysis and fermentation by bacteria, yeast, or fungi [35]. Microalgae such as *Chlorella vulgaris, Chlorococcum* sp., and *Chlorococcum littorale* and the cyanobacterium *Arthrospira platensis* (also called *Spirulina*) are frequently used in these processes (e.g., [36]). Fermentation-related genes, genes coding pyruvate decarboxylase (*pdc* gene) and the alcohol dehydrogenase (*adh* gene) from the ethanogenic bacterium *Zymomonas mobilis* have been expressed in *Synechococcus* and *Synechocystis* using native and strong promoters (P*rbcLS* and P*psbAII*) [37, 38]. However, ethanol production was still low compared to the amounts produced by microbial fermentation, probably due to the low tolerance of cyanobacterial systems to ethanol stress [38–40].

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2.1.3. Biogas

An alternative to liquid biofuels is the use of gas, which has some advantages over the use of liquid biofuels such as improved energy conversion efficiency of substrates and lower emission of toxic gases to the environment [41]. Biogas can be produced from different biomass feedstocks, such as dedicated energy crops, algal biomass, food wastes, animal manure, agricultural residues, industrial wastes [42]. Through the use of microalgae, it is possible to generate different types of biogas either by anaerobic fermentation of biomass (methane) or through the reduction of protons due to their hydrogenase activity (hydrogen gas, H_2) [43].

Methane can be produced through anaerobic digestion of microalgae biomass by bacteria and archaea, in a process that involves various reactions (hydrolysis, acidogenic, acetogenesis and methanogenesis). However, the productivity levels correlate directly with the degree of cell wall disruption and with the solubilization levels of the organic cell compounds. Different physical, chemical, and biological pretreatments have been tested for yield optimization [44–46]. Some examples of microalgae in which anaerobic enzymatic digestion have been described are *Scenedesmus* sp. [47], *Rhizoclonium* sp. [48], *C. vulgaris* [49], *S. obliquus*, and *A. maxima* [50].

Biohydrogen is an attractive fuel alternative because its combustion produces no carbon byproducts and it is superior for electricity production by fuel cells. While algae are capable of forming biomass through photosynthesis, oxygen hinders this process by inhibiting the enzyme hydrogenase. It is for this reason that, under anaerobic and dark conditions, microalgae from the genera *Chlamydomonas, Scenedesmus, Lobochlamys*, and *Chlorella* have the ability to produce H_2 by the action of hydrogenase or other enzymes able to metabolize H_2 [51]. The genetic engineering of microalgae to modify the hydrogenase has failed to significantly increase the levels of H_2 produced [52]. Moreover, overexpression of this enzyme in *Chlorella* has only achieved modest increases in H_2 production [53]. Other more successful strategies have been based on the inhibition of photosystem II (PSII) in order to inhibit the production of oxygen [54, 55].

2.2. Microalgal and cyanobacterial biomass and valued-added nutrients

Biomass of microalgae and cyanobacteria has also been harvested to be used as human nutrient supplements, animal feed, and fertilizers for many centuries [10].

Edible microalgae and cyanobacteria include the chlorophyceae *Chlorella* sp. and *Scenedesmus obliquus* and cyanobacteria from the genera *Arthrospira, Nostoc, Spirulina*, and *Aphanizomenon*. Microalgae and cyanobacteria are a good source of protein (including essential amino acids), essentials polyunsaturated fatty acids (PUFA) like eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), carbohydrates, and other beneficial compounds for human and animal health, such as vitamins, astaxanthin, lutein, beta-carotene, chlorophyll, phycobiliprotein, and beta-1,3-glucan [56, 57].

Lipid metabolism has been mainly studied in the green microalga *C. reinhardtii* through genomic data and genetic tools. A general overview of these discoveries can be found in several scientific reports [58, 59]. However, the molecular mechanisms involved in regulatory pathways in algae are still poorly understood.

Algal pigments, such as carotenoids, are widely exploited by the food industry for the pigmentation of salmon, chicken, trout, and shrimps; by the nutraceutical industry for their antioxidant and other health benefits; by the cosmetics business for their skincare and anti-aging properties. Algal pigments are also used for the fortification of foods and beverages. The most popular pigments are chlorophyll, beta-carotene, alpha-carotene, lutein, lycopene, zeaxanthin, and astaxanthin. Astaxanthin has surged in the nutraceutical market because its unusual antioxidant capacity and several health benefits [60]. Even though the main supply of astaxanthin used for the pigmentation of salmon is now 95% of synthetic origin, there has been a growing trend toward using natural ingredients. Thus, the demand for natural astaxanthin from the green microalgae *Haematococcus pluvialis* has grown considerably [61].

3. Gene expression and its constraints for microalgae and cyanobacteria molecular cell physiology

The basic genetic construct used to express a specific gene must consider the proper recognition of the DNA structures related to transcription and translation by the host cell, that is, promoters, ribosome binding sites, the codon usage to translate the target gene, and transcription terminators. Therefore, there are many constraints that need to be considered during the planning of genetic modifications.

3.1. Transcriptional control

3.1.1. Promoters

The selection of the right promoter will depend on diverse variables such as the organism to be modified (cyanobacteria, microalgae), the gene expression levels required (constitutive, induced expression, strong expression), or the target DNA that can belong to different cellular compartments in eukaryotes.

In eukaryotes, native promoters have been extensively described for nuclear and organelle transformation. Their main advantage is the correct recognition by the enzymatic transcriptional machinery of the microalgae. Some examples of these promoters are as follows: (i) for *C. reinhardtii*, the 5′untranslated region (5′-UTR) of the RuBisCO small subunit gene *rbc*S2 and heat shock protein 70A gene *hsp*70A [62, 63]; and (ii) for *Nannochloropsis*, the 5′-UTR of violaxanthin/chlorophyll *a*-binding protein (VCP) genes and the ß-tubulin promoter [64–66]. Heterologous promoters have also been used in different types of microalgae such as viral promoters 35S, CaMV35S, SV40, and CMV. One of the advantages of these promoters is that they can be recognized in some microalgal systems such as *C. reinhardtii* [67], *D. salina* [68], *D. bardawil* [69], *C. vulgaris* [70], *Platymonas subcodiformis* [71], *Nannochloropsis* sp. [70], *H. pluvialis* [72], and *Phaeodactylum tricornutum* [73].

For cyanobacteria, there is a set of constitutive and inducible native promoters used in biotechnological studies to express heterologous genes or increase the expression of native

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ones. Some examples of these promoters are as follows: (i) the copper-inducible promoter P*petE* [14], (ii) the nitrate-inducible promoter P*nirA* [74], and (iii) the constitutive promoters P*psbA2* and P*rcbL* [13]. Genetic transformations for biotechnological purpose considering inducible promoters have gained a remarkable interest due to the advantage to obtain high yield of biomass before interfere the normal cell function and growth with the expression of the gene(s) of interest. However, efficient controllable promoters in cyanobacteria are scarce [75]. Additionally, the effectiveness of heterologous promoters in cyanobacteria is very low and successful examples using lac-regulated and *tetR*-regulated promoters need to include critical modifications to fit the sequences to the transcriptional machinery of cyanobacteria [76].

3.2. Translational control

3.2.1. Codon usage

Different organisms usually bear particular codon usage patterns. Therefore, when a gene from one species is cloned and expressed in another, some codons might be rare in the new host, leading to poor translation efficiency or starvation for certain amino acids and consequently lose the cell's fitness [77]. Specific variations in codon usage are often cited as one of the major factors impacting protein expression levels. The presence of rare codons that correlate with low levels of their endogenous tRNA species in the host cell can decrease the translation rate of target mRNAs.

Genes in cyanobacteria show a bias in the use of synonymous codons [78–81]. The importance of codon optimization in algal genetic applications also been seen in microalgae such as *C. reinhardtii* [77], *Gonium pectorale* [82], and *Porphyra yezoensis* [83]. In *C. reinhardtii*, codon usage was reported to affect the expression of some foreign genes such as GFP [77]. Sequencing studies and expression profiles of microalgae have clarified these issues, thereby allowing optimization of codon usage at the level of expression in the nucleus [82] and in the chloroplasts [84–87].

3.3. Barriers for transfer foreign DNA into microalgae and cyanobacteria

There are several obstacles to be faced when trying to insert exogenous DNA into eukaryotic and cyanobacterial systems. The first is physical barriers such as cell wall, plasma membrane, nuclear membrane, mitochondrial membrane, and chloroplast membrane.

To overcome these "natural barriers" diverse experimental strategies have been designed, such as the generation of spheroplasts (cells without cell wall) for eukaryotic microalgae by mechanical disruption or enzymatic treatments [88, 89]. However, problems of protoplast viability and low post-transformation recovery have been described in some systems. Other strategies include the application of multipulse or high electrical voltage for the disruption of the algae extremely rigid cell walls (e.g., *Nannochloropsis* sp.) and the entry of exogenous DNA [64–66, 90]. The low number of transformed cells recovered is one of the problems with this type of strategy. This is probably due to the high stress conditions to which the cells are subjected and the difficulty of foreign DNA integration into the genome found in many

eukaryotic systems. Another obstacle is the transient insertion of the exogenous DNA and the eventual loss of the phenotype of interest over time. This is due in part to the lack of understanding of the DNA insertion mechanisms into the cell compartments of some microalgae.

Cyanobacteria have a special envelope formed by the outer membrane, a thick peptidoglycan layer, the s-layer, and frequently an exopolysaccharide EPS envelope [91]. The EPS can represent a high proportion of the total cell dry biomass of environmental or extremophile cyanobacteria (e.g., *Nostoc calcicola*, [92]) and can be a barrier for DNA uptake in cyanobacteria. Several protocols for reducing EPS prior to transformation have been implemented for those cyanobacteria such as: (i) growth conditions under constant agitation, (ii) culture media supplemented with low concentrations of saline sodium citrate (SSC) [93], (iii) pumping the cells out of the sheath using a syringe [94], and (iv) treatment by agitation with concentrated NaCl and several washing steps [95].

In addition, cyanobacteria have mechanisms of defense against exogenous DNA that hinders the transformation process. The majority of filamentous cyanobacteria and some unicellular representatives contain restriction enzymes that can degrade the transferred DNA [96]. Strategies to improve the DNA transfer efficiency include the following: recipient strains with inactivated endonucleases [97]; deletion of the restriction sites from the foreign DNA [98]; or more often, the protection of the foreign DNA by methylation, using specific methylases encoded in helper plasmids, prior to transformation of the cyanobacteria [99–101].

4. Planning and strategies for genetic modifications in microalgae

4.1. Transformation of eukaryotic microalgae

Transformation in eukaryotic systems has several advantages when compared to cyanobacteria. For example, the functional recombinant protein needs, in many cases, the post-translational modifications only developed by the eukaryotic machinery, for example, protein folding assisted by chaperones in chloroplasts or specific protein glycosylation in the endoplasmic reticulum and Golgi apparatus (See [102], and references therein). Furthermore, the organellar machinery (i.e., mitochondria and chloroplast) gene expression resembles that of prokaryotic cells. Thus, genetically modified eukaryotes can produce a versatile range of proteins, including toxic proteins with prospects in cancer therapies [103].

Among examples of successfully transformed microalgae are *C. reinhardtii* [104], *Cyanidioschyzon merolae* [105], *Ostreococcus tauri* [106], and *Nannochloropsis* sp. [64, 90].

The integration of genes for nuclear transformation of microalgae occurs at random locations, with homologous recombination occurring at very low frequency [107]. On the contrary, plastidial transformations are based on homologous recombination similar to what is described for cyanobacteria.

Different strategies for inserting foreign DNA into microalgae have been described (biolistics, electroporation, natural transformation, random mutation) [102, 108]. However, each micro-

algal species has its own morphological, structural, and physiological characteristics, making the development of standard protocols for all species difficult.

4.1.1. Biolistic transformation

Biolistics is one of the most effective methods of transformation which has been mainly used for the insertion of genes into plastids such as chloroplast. This method makes use of DNA-coated heavy metal (mostly gold) microprojectiles and allows transformation of almost any type of cell. Its advantages are its versatility. Different types of DNA constructs can be used on metal particles, and they can transform various types of organelles (nucleus, mitochondria, and chloroplast). Also, high amounts of recombinant proteins can be accumulated into plastids such as chloroplasts, which have high stability due to biochemical differences with the cytoplasm. Among the difficulties is the high cost of the equipment and the inability to express proteins with complex translational modifications. This method has been applied so far in the transformation of many algal species such as the diatoms *Cyclotella cryptica* and *Navicula saprophita* [109], Chlorophyta such as *C. reinhardtii* [110], *Volvox carteri* [111], *Chlorella sorokiniana* [112], *Chlorella ellipsoidea* [113], *Chlorella kessleri* [114], *H. pluvialis* [72], *P. tricornutum* [115], and *G. pectorale* [82].

4.1.2. Electroporation

This technique has been used for many years for the transformation of numerous prokaryotic and eukaryotic cells. The great advantages of this technique are its versatility, simplicity, and high efficiency.

Electroporation conditions are dependent on the type of microalgae and structural characteristics of each one. For example, *Nannochloropsis* microalgae species that have a rigid cell wall need the application of high electrical voltage in order to allow the entry of foreign DNA into the cell nucleus. However, these very demanding conditions have reported low rates of recovery of transformed clones.

This technique has been successfully used in some microalgae such as *C. reinhardtii* [116], *C. merolae* [105], *D. salina* [117], *C. vulgaris* [118], and *Nannochloropsis* sp. [64–66, 90, 119].

4.1.3. Random mutagenesis

Mutation followed by selection of favorable phenotypes has been used for crop plants, and some promising strategies are now beginning to emerge for microalgae. Random mutation strategies applied to microalgae include the use of mutagenic chemicals and radiation. Among the disadvantages of these techniques is the difficulty in identifying the location in the genome of these mutations in the genome and the difficulty of controlling the number of mutational events per experiment. One way to solve these problems is to randomly mutagenize the genome of microalgae using genetic elements such as transposons of random insertion. This

methodology allows for easy recognition of the DNA incorporated using amplification molecular tools [120].

Microalgae that have been transformed with these methods include *Isochrysis affinis galbana* [121], *Nannochloropsis* sp. [122], *C. reinhardtii* [120], *Pavlova lutheri* [123], *Scenedesmus dimorphus* [124], *C. sorokiniana* and *S. obliquus* [125], and *Parietochloris incisa* [126].

4.2. Transformation of cyanobacteria

Unicellular and multicellular cyanobacteria have a varying number of genome copies per cell (variable polyploidy), even between members of the same strain under different physiological conditions through their life cycle [127]. This characteristic complicates considerably the full segregation of genome-targeted mutations. However, the scientific literature shows many examples of genetically modified cyanobacteria, especially unicellular cyanobacteria, mainly through natural transformation, conjugation, and electroporation. DNA bombardment (biolistics) is less frequent, but it has been used [95, 128].

4.2.1. Natural transformation

Some cyanobacteria are naturally competent, being able to be transformed by natural incorporation of foreign DNA from the environment. This ability is a simple and rapid way to introduce exogenous DNA into cyanobacteria and has been commonly used to transform cyanobacterial strains through gene replacement by double crossover homologous recombination, which is attained by the arrangement of two homologous sequences flanking the exogenous DNA of interest. Example of natural transformable model strains are as follows: *S. elongatus* PCC 7942 [129, 130], *Synechococcus* sp. PCC 7002 (or *Agmenellum quadruplicatum*) [131, 132], *Synechocystis* sp. PCC 6803 [133–135], and *Thermosynechococcus elongatus* BP-1 [97, 136, 137].

The exogenous DNA can be a linear or be carried on a plasmid prepared using standard procedures. The cyanobacterial cells with this ability are easily transformed during the logarithmic growth phase; while on stationary growth phase, they show reduced transformation efficiencies [131, 135, 137, 138].

As natural transformation triggers DNA fragmentation [137, 139, 140], this procedure is not recommended for single recombination or intact replicating plasmids. For these, conjugation or electroporation is preferred.

4.2.2. Electroporation

Electroporation is commonly used for transferring plasmids into diverse cyanobacterial cells (see further in [141]). Electroporation promotes single rather than double recombination events. It is worth noting that mutagenic events and events of recombination in nonspecific loci are frequent when electroporation is used to transform cyanobacteria [137, 140].

4.2.3. Conjugation

The most common technique for transforming cyanobacteria has been conjugation. Conjugation is the transfer of plasmid DNA from a donor (commonly *E. coli*) to a recipient cell through direct contact. This technique has been widely used in the filamentous-type strains *Nostoc* PCC 7120 [98], *N. punctiforme* ATCC 29133 [142–144], and *Anabaena variabilis* ATCC 29413 [145], as well as for the unicellular strains *S. elongatus* PCC7942, *Synechococcus* sp. PCC6301 [140], and *Synechocystis* PCC 6803 [146, 147].

Because several cyanobacteria, especially filamentous cyanobacteria, contain endogenous restriction endonucleases, the design of the plasmids must avoid predicted target sites of native restriction enzymes [98], or must be previously methylated to prevent the action of them [99, 100]. The most frequent conjugation procedure typically includes two different *E. coli* strains (e.g., [95, 148]). One carries the conjugal plasmid, and the other the helper plasmid(s) and the cargo plasmid to be transferred to the cyanobacterium. Cargo plasmid contains the DNA information to be transfer into the cyanobacteria and can be either a replicating plasmid or an integrative plasmid. It must also carry an "origin-of-transfer" (oriT) from a fragment of RP4 or relative plasmids, and a *mob* gene encoding a nickase, which recognizes the oriT and provides a single-stranded DNA for transfer [98, 146]. Alternatively, the *mob* gene may also be provided in *trans* in the helper plasmid, which also carries genes encoding methylases to protect the DNA of interest from the mentioned degradation by restriction enzymes.

4.2.4. Plasmids vectors

Plasmids vectors are one of the most common tools for transferring foreign DNA containing the information for the genetic manipulation of cyanobacteria. They can be classified based on the type of transformation into (i) integrative vectors, which modify the genomic information of the target organism by recombination or transposition (*cis*-transgene expression); and (ii) replicative or shuttle vectors, which are plasmids that can replicate themselves with the aim of expressing genes without any alteration to the host's genome (*trans*-transgene expression).

cis-transgene expression, achieved through an integrative vector by double homologous recombination, is the most frequent approach in cyanobacterial research, either for biotechnology or basic science studies. Plasmids introduced by conjugation are circular and a single recombination allows integration of the selective marker. Alternatively, a strategy to select less frequent double recombination events has been implemented based on suicide plasmids [149]. For gene knockout and knockin, researchers prefer these suicide plasmids to ensure the integration of the exogenous DNA and avoid the gene expression from the plasmid (e.g., [150]). The suicide plasmid contains an antibiotic resistance cassette and a *sacB* gene, which encodes levansucrase, an enzyme that converts sucrose to levans (a toxic fructose polymer). Only the antibiotic resistance gene is planned into be integrate in the chromosome by the double homologous recombination, while the *sacB* gene will remain in the plasmid. Double recombinant mutants are selected first using the antibiotic resistance, and then, the survival mutants are placed onto media with 5% sucrose to kill the cells containing the suicide plasmid or the single recombination. The *sacB*-antibiotic resistance cassette is also used to select "markerless"

chromosomal mutations [38]. In this case, the complete *sacB*-antibiotic resistance cassette fragment is integrated into the chromosome and mutants are selected for resistance to the antibiotic. A second transformation of the survival mutants is made with a fragment designed to replace the *sacB*-antibiotic resistance sequence. The survival mutants growing on culture media with sucrose are the ones containing the double transformation, although PCR corroboration is needed.

Gene expression can be enhanced through shuttle plasmids. The recognition of the replication origin by the host cell is essential for shuttle vectors, and in cyanobacteria, there are few studies using this type of plasmids, mainly based on the self-replicating base RSF1010 plasmid [146, 151, 152]. Recently, Taton and colleagues [152] offers an online bioinformatic tool to assembly cyanobacterial plasmids, including self-replicating ones.

4.3. New transformation strategies for microalgae and cyanobacteria

The insertion of exogenous genetic material using organisms such as *Agrobacterium tumefaciens* has also been reported in some microalgae. This strategy allows efficient insertion of genetic material into the nuclear DNA and expression of the respective reporter genes. Among the target organisms, we found species of *Chlamydomonas* [67], *Dunaliella* [69], *Chlorella* [153], *Schizochytrium* [154], *H. pluvialis* [72], and *Nannochloropsis* sp. [70]. Examples of natural transformation have been recently described for diatoms *P. tricornutum* and *Thalassiosira pseudonana*, in which, episomal vectors were inserted by conjugation with *E. coli* [155]. The ability to use such strategies in other microalgae is something that should be evaluated on a case-by-case basis.

The CRISPR/Cas system, a heritable adaptive immunity system [156–158], has been adapted for targeted gene editing in mammalian, plant, fungal, and bacterial hosts [159]. In addition, silencing of multiple genes through a CRISPR-interference (CRISPRi) platform that contains a nuclease-deficient Cas9 has also been reported [160, 161]. The use of the CRISPRi system for gene silencing in *Synechocystis* sp. PCC 6803 was described recently [162]. This technique was able to repress the expression (or at least lower the expression) of several genes in this model cyanobacterium, including genes related to the formation of carbon storage compounds during nitrogen starvation (glycogen and polyhydroxybutyrate) and genes probably involved in long chain alkane production (aldehyde reductases and dehydrogenases) [162]. However, additional studies are needed to evaluate the functionality of these promising systems as tools for genetic engineering in others cyanobacteria and eukaryotic microalgae.

4.4. Selection and reporter markers genes

The use of selectable marker genes is normally required in all experiments that aim to generate stable transgenic algae because only a very low percentage of treated organisms are successfully transformed. Selectable markers are often antibiotic resistance genes, which are dominant markers as they confer a new trait to any transformed target strain of a certain species, independent of the respective genotype.

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Numerous genes that confer resistance to various antibiotics and herbicides have been reported in microalgae and cyanobacteria (antibiotics such as zeocin, hygromycin, bleomycin, chloramphenicol, kanamycin, phleomycin and the herbicides sulfounylurea, norflurazon) [163] allowing easy selection of transformed transgenic organisms (dominant selection markers). Among the known disadvantages of such markers is that their sensitivity is specific to each microalgae.

Another class of transformation markers are reporter genes that allow selecting transformant strains based on a particular phenotypic characteristic conferred by this gene. Among these are luminescent, fluorescent, and chromogenic proteins (such as sfCherry, mCherry, GFP, GUS, SHCP, and luciferase) are currently been used to evaluate gene expression levels in cyanobacteria and eukaryotic microalgae [65, 66, 84, 119, 164, 165]. The combination of these gene reporters with optical techniques such as flow cytometry coupled to cell sorting could make this process ecologically friendly and efficient for the selection of promising phenotypes (e.g., higher gene expression of commercially interesting compounds). The use of reporter genes that provide detectability through optical methods has been recently implemented for the selection of successful transformants, especially in eukaryotic microalgae [119, 166].

5. Conclusions and future outlook

Microalgae and cyanobacteria are true sources of different types of natural compounds with applications as diverse as: human food, animal feed, aquaculture, chemicals and pharmaceuticals, pigments, diatomites, fertilizers, wastewater treatment, cosmetics and fuels. Hence, better understanding of their physiological, morphological, biochemical, and molecular characteristics could be profitably applied in biotechnology.

Nowadays, there are a limited but increasing number of transformed microalgae. The technological advances and increasing collection of genomes sequenced, together with the identification and characterization of new molecular elements within the cell (e.g., promoters, codon usage, terminators, plasmids, selection markers, and reporter genes) may reduce unplanned gene expression deficits and achieve a well-developed, photosynthetic cellular platform for the implementation of useful and sustainable applications. Furthermore, the discovery of these new elements allows the development of new transformation strategies and the re-evaluation of genetic elements to study the functionality of key genes.

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