Transformation of the filamentous Cyanobacterium *Phormidium lacuna*

Electroporation versus Natural Transformation

Zur Erlangung des akademischen Grades eines

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Fabian Nies (Karlsruhe, 05.01.2019)

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Zusammenfassung

Der Großteil der aktuellen, auf Kohlenstoff basierenden Waren wird entweder aus pflanzlichen Rohstoffen oder aus Erdöl gewonnen. Die damit verbundene Nachfrage nach Ressourcen steigt auf Grund der wachsenden Erdbevölkerung und den allgemeinen Bestrebungen zur Verbesserung des Lebensstandards. Im Gegensatz dazu sind die Verfügbarkeit von Agrarflächen und fossilen Energiereserven begrenzt. Außerdem wird der Anstieg von CO_2 in der Atmosphäre seit dem Beginn der industriellen Revolution im 19. Jahrhundert als Hauptbeitrag des Menschen zur globalen Erwärmung angesehen. Es gibt einige Strategien um die Abhängigkeit von fossilen Kohlenstoffquellen zu reduzieren, wie etwa die Entwicklung von regenerativen Energien oder erhöhte Energieeffizienz.

Der Einsatz von Cyanobakterien in der Biotechnologie ist eine weitere Strategie, die das Potential besitzt Landwirtschaft und Erdölindustrie als Quelle für kohlenstoffbasierte Produkte zu ersetzen. Produkte, die sich von Cyanobakterien oder Pflanzen ableiten, führen nicht zur Erhöhung des CO_2 Gehalts in der Atmosphäre. Im Vergleich zu Landpflanzen ist die Kultivierung von Cyanobakterien nicht abhängig von fruchtbarem Land und viele Arten der Cyanobakterien weisen höhere Wachstumsraten als Pflanzen auf. Außerdem sind einige Cyanobakterien genetisch manipulierbar, wodurch die Synthese natürlicher Produkte verstärkt werden kann oder die rekombinante Produktion von neuen Stoffen möglich ist. Die mögliche Produktpalette reicht von preiswerten Verbindungen, wie Biokraftstoffen, bis hin zu hochwertigen Produkten, wie pharmazeutischen Wirkstoffen.

Auch wenn es im Bereich der rekombinanten Biotechnologie mit Cyanobakterien schon kommerzielle Anwendung gibt, befindet sich das gesamte Feld dennoch in seinen Kinderschuhen. Während im Bereich der metabolischen Optimierung, der molekularen Werkzeuge und der Bioreaktorentwicklung in den letzten Jahren und Jahrzehnten deutliche Fortschritte gemacht wurden, ist die Etablierung von neuen Organismen nach wie vor eine Herausforderung. Die Forschung konzentriert sich größtenteils auf wenige, leicht transformierbare Modelorganismen der Ordnungen Synechococcales und Nostocales, da die Etablierung von Transformationsprotokollen für neue Stämme und Arten oft eine Herausforderung darstellt. Allerdings wird die rekombinante Biotechnologie mit Cyanobakterien ohne ein breites Spektrum an nutzbaren Stämmen möglicherweise hinter ihrem Potential zurück bleiben, da vorteilhafte Eigenschaften von vielversprechenden Kandidaten nicht genutzt werden können.

Diese Arbeit setzt sich mit der beschriebenen Limitierung auseinander, indem ein verlässliches und effizientes Transformationsprotokoll für *Phormidium lacuna* etabliert wurde. Diese Art wurde kürzlich von unserer Arbeitsgruppe als vielversprechend für die cyanobakterielle Biotechnologie charakterisiert. Da die Gattung *Phormidium* bislang noch nicht für genetische Manipulation zugänglich war, war es das Ziel dieser Arbeit ein Transformationsprotokoll zu entwickeln und das Potential für die Biotechnologie an Hand der rekombinanten Ethanolsynthese zu charakterisieren. Die rekombinante Produktion von Ethanol ist gut charakterisiert innerhalb der cyanobakteriellen Biotechnologie und ermöglicht den Vergleich mit anderen Cyanobakterien hinsichtlich Produktivität.

Bei der Etablierung eines Protokolls zur Elektroporation wurde deutlich, dass *Phormidium lacuna* natürlich transformierbar ist. Natürliche Transformation ist bislang nur für wenige Stämme der Cyanobakterien beschrieben und dies ist der erste Bericht für die Ordnung Oscillatoriales. Natürliche Transformation erlaubt verlässlichen und effizienten Gentransfer mittels homologer Rekombination in *Phormidium lacuna*. Die Integration des Selektionsmarkers *kanR* ins Genom von *Phormidium lacuna* vermittelt eine deutliche Resistenz gegenüber Kanamycin (bis zu 14,3 mg/ml). Die *kanR* Sequenz verteilt sich sehr schnell in allen Genomkopien: Nach zwei Kulturzyklen nach der ersten Kultivierung der entsprechenden Transformanten war die *kanR* Sequenz in allen Genomkopien einer Zelle vorhanden.

Phormidium lacuna Transformaten für die rekombinante Ethanolproduktion wurden generiert indem die codierenden Sequenzen für die Enzyme Pyruvatdecarboxylase und Alkoholdehydrogenase ins Genom integriert wurden. Die Ethanolproduktion wurde über die Ethanolkonzentration im Kulturüberstand mittels Gaschromatographie nachgewiesen. Allerdings konnte keine erhöhte Ethanolproduktion von Transformanten im Vergleich zum Wildtyp von *Phormidium lacuna* nachgewiesen werden. Mögliche Gründe hierfür und geeignete Schritte in Richtung einer biotechnologischen Anwendung von *Phormidium lacuna* wurden diskutiert.

Der unerwartete Fund der natürlichen Transformation für *Phormidium lacuna* ist möglicherweise ein Hinweis darauf, dass die Fähigkeit zu natürlicher Transformation weiter unter den Cyanobakterien verbreitet ist, als bislang angenommen wurde. Um diese Fragestellung zu adressieren wurden der Fund der natürlichen Transformation in dieser Arbeit und die weiteren Beispiele, die in der Literatur beschrieben sind, mit den Cyanobakterien im Allgemeinen verglichen. Basierend auf der Homologie zu Proteinen, die an der natürlichen Transformation beteiligt sind, wurden cyanobakterielle Stämme vorgeschlagen, die potentiell transformierbar sind.

Der Fund der natürlichen Transformation in dieser Arbeit hat viele Implikationen. Erstens: *Phormidium lacuna* ist nun effizient natürlich transformierbar, was eine essentielle Voraussetzung für die weiteren Arbeitsschritte mit diesem Organismus in Bereich der rekombinanten Biotechnologie darstellt. Zweitens: Da *Phormidium lacuna* leicht transformierbar ist und homozygote Transformanten in kurzer Zeit generiert werden können, bietet sich dieses Bakterium als vielversprechender Modellorganismus der filamentösen Cyanobakterien ohne Heterocysten an. Drittens: Der Fund von natürlicher Transformation in einer neuen Ordnung der Cyanobakterien weiß vermutlich daraufhin, dass natürliche Kompetenz eine weit verbreitete Eigenschaft innerhalb der Cyanobakterien ist und dass möglicherweise deutlich mehr Stämme zugängliche für rekombinante Biotechnologie sind.

Abstract

Our present consumption of organic commodities mostly relies on plant material or mineral oil. The related demand for resources is increasing due to the increase of world's population and overall efforts for improved standards of living. This is in conflict with the limited availability of arable land and decreasing reserves of fossil fuels. Furthermore, the release of CO_2 into the environment by the increasing use of fossil energy since the industrial revolution in the 19^{th} century is regarded as main anthropogenic contribution to the global climate change. Several strategies exist to decrease the dependency on mineral carbon sources like the development and use of alternative energy sources and increasing energy efficiency.

The use of cyanobacteria in biotechnology is an additional strategy that has the potential to supplement agriculture and fossil fuel industry as source of carbon based compounds. Products originating from cyanobacteria and plants are not related to the release of new CO_2 into the atmosphere. In comparison to plants the cultivation of cyanobacteria is not dependent on arable lands and many cyanobacterial species show higher growth rates than plants. Furthermore, several cyanobacteria are accessible for genetic manipulation, which allows to improve the synthesis of original products or to facilitate the production of recombinant compounds. The range of possible products from cyanobacterial biotechnology encompasses low value products like biofuels up to high value compounds for pharmaceutics.

Although there are already commercial companies in the field, application of cyanobacterial biotechnology is still in its infancy. While remarkably progress has been made in the field of metabolic engineering, molecular tools, and bioreactor design in the last decades, the establishment of new organism in the field is still challenging. Research mainly focuses on the few easily transformable model organisms of the orders Synechococcales and Nostocales since the establishment of transformation protocols for new strains and species is often challenging among the phylum of cyanobacteria. But without a broad spectrum of accessible strains for cyanobacterial biotechnology, this field may stay behind its potential, since beneficial properties of potential producer strains are not useable.

This study addresses this limitation by the establishment of a reliable and efficient transformation protocol for *Phormidium lacuna*. This species was characterized with promising traits for cyanobacterial biotechnology by our workgroup recently. As the genus *Phormidium* was not accessible for genetic manipulation so far, the aim of this study was to establish a protocol for gene transfer into *Phormidium lacuna* and to characterize its potential for recombinant biotechnology with the heterologous production of the biofuel ethanol. The recombinant production of ethanol is well characterized in cyanobacterial biotechnology and allows the comparison with other cyanobacteria in terms of productivity.

During the establishment of gene transfer into *Phormidium lacuna* via electroporation, it was discovered that this organism is capable of natural transformation. The process of natural

transformation has only been described for a few cyanobacterial strains before and this is the first report for the order Oscillatoriales. Natural transformation allows reliable and efficient gene transfer into *Phormidium lacuna* via homologous recombination. Integration of the selection marker *kanR* into the genome mediates a considerably high resistance against kanamycin for *Phormidium lacuna* transformants (up to 14.3 mg/ml). The *kanR* sequence segregates very fast in the genome: After two cultivation cycles following the first cultivation of *Phormidium lacuna* transformants in suspension culture, the *kanR* sequence was present in all genome copies in a cell.

Phormidium lacuna transformants for ethanol production were generated by the integration of the coding sequences for pyruvate decarboxylase and alcohol dehydrogenase into the genome. Ethanol production was monitored by the ethanol content in culture supernatant using gas chromatography. However, no increased ethanol production could be demonstrated for any transformant in comparison to the wild type of *Phormidium lacuna*. Probable reasons for this finding and suitable further steps towards the biotechnologically application of *Phormidium lacuna* were discussed.

The unexpected finding of natural transformation in *Phormidium lacuna* indicate that natural transformation is a more common trait among the cyanobacteria than previously thought. To address this hypothesis, the findings of natural transformation in *Phormidium lacuna* in this study and other examples of natural transformation in literature were set into relation to the phylum of cyanobacteria. With a homology based study on the sequences of proteins, which are involved in natural transformation, several other cyanobacterial strains were predicted by bioinformatics to be potentially naturally transformable.

The finding of natural transformation has many implications. First, *Phormidium lacuna* is now efficiently genetically manipulable, which is an essential prerequisite for further work with this organism in respect to recombinant biotechnology. Second, since *Phormidium lacuna* is easily transformable and also homozygous transformants can be generated in short time, this organism might be an interesting model for filamentous cyanobacteria without heterocysts. Third, the finding of natural transformation in a new order of cyanobacteria might indicate that natural transformation is a more common trait among cyanobacteria and more strains might be easily accessible for recombinant work.

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List of abbreviations

ADH	alcohol dehydrogenase
AmpR	ampicillin resistance operon
BLAST	basic local alignment search tool
CNT	candidates to test for natural transformation
EP	electroporation
EPS	extracellular polymeric substances
E value	expect value
GC	gas chromatography
GFP	green fluorescent protein
KanR	kanamycin resistance operon
NCC	naturally competent cyanobacteria
NT	natural transformation
NTF	natural transformation factor
OD	optical density
ORF	open reading frame
PDC	pyruvate decarboxylase
PHB	polyhydroxybutyrate
UTR	untranslated region
WT	wild type

1 Introduction

In recent years research in cyanobacteria gained an increasing application-respect. Besides long uses as dietary supplement of cyanobacteria like *Arthrospira*, prominent under its former name *Spirulina*, or pigment source (Ciferri, 1983, Leema *et al.*, 2010), today's main focus is on the production of renewable energy sources or bulk chemicals. But also in respect of pharmaceutically relevant compounds cyanobacteria are under investigation (Vijayakumar & Menakha, 2015). The concept of converting CO_2 and solar energy into valuable products has been a motor in cyanobacterial biotechnology research, especially in consideration of climate change and limited fossil energy resources. Besides isolating naturally produced compounds such as fatty acids (Guedes *et al.*, 2011) and hydrogen (Ananyev *et al.*, 2008), especially recombinant production has raised interest. By genetic engineering the energy- and carbon-flow is redirected towards a desired product by either improving existing or integrating new metabolic pathways. Most common are the investigations on the recombinant production of ethanol by cyanobacteria, which was first demonstrated in 1999 by Deng and Coleman. These authors could demonstrate ethanol production in *Synechococcus elongatus* PCC 7942 by the heterologous expression of the enzymes pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) from *Zymomonas mobilis* (Deng & Coleman, 1999). Since then the production rate of ethanol was optimized from 0.0082 g L^{-1} d^{-1} to 0.26 g L^{-1} d^{-1} (Dienst *et al.*, 2014) and 0.55 g L^{-1} d^{-1} (US patent: 20140178958). Production rates for ethanol and other products are summarized in several reviews (Angermayr *et al.*, 2015, Dexter *et al.*, 2015).

There are three major aspects to increase the potential of recombinant cyanobacterial biotechnology, which all interact with each other: metabolic engineering, bioreactor design, and the choice of organism (Figure 1). The yield of product can be influenced by metabolic engineering via expression rate and efficiency of the respective enzymes (see below). Export of the product can be achieved by heterologous expression of corresponding transports (Abramson *et al.*, 2016). Furthermore, carbon flux from CO_2 towards product can be mediated by deletion or inhibition of competing pathways (see below). Also long term genetic stability of producer strains is a challenge (Jones, 2014). Crucial points for bioreactor design are enabling high product yields and effective subsequent product separation. Product formation is mostly connected to efficient cell propagation. Important factors are light and CO_2 distribution, temperature regulation, and nutrient supply in a cost efficient manner (see below). The general decision whether a selected bacterial strain is cultivated in suspension culture or adherent in a biofilm is of central importance for bioreactor design. There are some well characterized unicellular and filamentous cyanobacteria, which are genetically accessible and are also used as model organisms in basic research (see below). These species are often considered when it comes to the choice of organism for recombinant biotechnology. In contrast, the majority of cyanobacteria are not genetically accessible. While the number of available genomes increases rapidly in the last years the establishment of transformation protocols for new organisms proceeds much slower and is still a bottleneck. Thus, at present the choice of cyanobacteria for recombinant biotechnology is limited and

the use of new, potentially promising organism is especially restricted by the elaborate and risky establishment of effective transformation protocols.

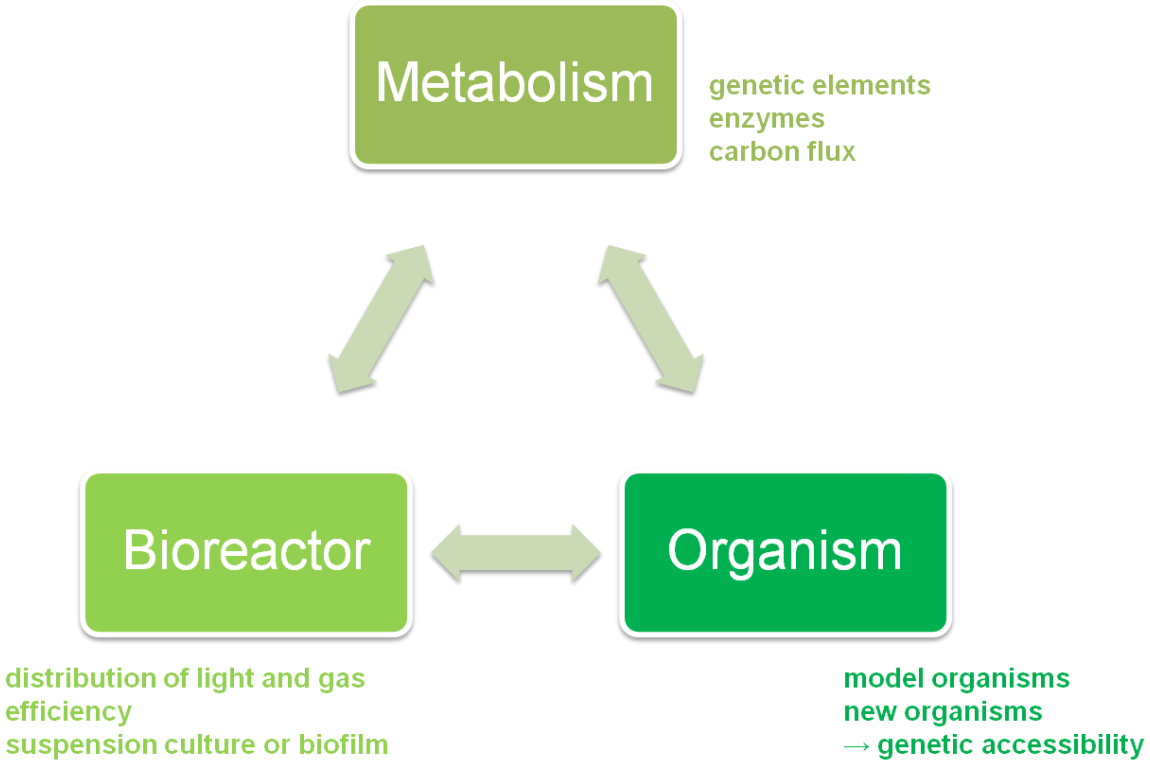


Figure 1: Recombinant biotechnology in cyanobacteria. Three central fields to increase the potential are metabolic engineering, bioreactor design, and the choice of organism.

1.1 Microalgal Biotechnology

The term microalgae describes microorganisms that are capable of oxygenic photosynthesis. The members of this group are defined by their lifestyle and not by close relationship. Several groups of photosynthetic eukaryotes and the prokaryotic cyanobacteria are included by this definition. Microalgae are of interest for biotechnological application because only light, CO_2, and inorganic salts are required for cultivation due to their photoautotrophic lifestyle. In contrast to land plants the cultivation of microalgae is not reliable on arable land and growth rates are much higher for several representatives (Medipally *et al.*, 2015, Sarsekeyeva *et al.*, 2015).

Microalgal cultivation systems can be classified as open pond and closed photobioreactors. While open pond (natural and artificial) are easy and cheap to construct, photobioreactors enable more controlled cultivation and the closed system protects against contaminations (Hamed, 2016). Photobioreactors can compensate higher costs with higher efficiencies. The main challenges are the distribution of CO_2 and light in an efficient manner (Posten, 2009). Light can only penetrate short distances in dense microalgal suspensions and under sunlight irradiation surface cells can be challenged by photoinhibition (Huang *et al.*, 2017). Several bioreactor designs are under investigation in order to facilitate even distribution of light and CO_2 while reducing energy input: Stirred tank,

vertical or horizontal tubular and flat panel photobioreactors are used for suspension culture in numerous variations (Singh & Sharma, 2012, Gupta *et al.*, 2015). While suspension culture systems are more common, also biofilm based photobioreactors are investigated. Advantages of this system are higher cell densities per volume and a more efficient separation of cells and medium but the distribution of growth factors may be impaired by the biofilm (Li *et al.*, 2017).

In respect of microalgae as renewable energy source two principles of production are possible. Either the biomass of the complete cell (or subsections like lipids or polysaccharides) are used for biofuel production or cells produce biofuels directly by genetically introduced pathways. For instance, the green algae *Botryococcus braunii* can reach lipid contents of above 80% of its dry mass (Wolf, 1983). But also other microalgae can reach high lipid contents (Araujo *et al.*, 2011). Lipids and fatty acids can be used to produce biodiesel via (trans-) esterification with short-chain alcohols. Photosynthetic microorganisms can be used as alternative to mineral oil not only in respect of biofuel production but also as source for bioplastics. Several cyanobacteria can be used to accumulate polyhydroxybutyrate (PHB) under unfavorable growth conditions like nitrogen starvation (Troschl *et al.*, 2017). Native production of valuable compounds can be increased or the production of new compounds can be enabled by recombinant DNA techniques. The production of several potential fossil fuel substitutes are reported like 1-butanol (Lan & Liao, 2011), isobutanol and isobutyraldehyde (Atsumi *et al.*, 2009), and isoprene (Lindberg *et al.*, 2010). Also lactic acid can be produced as precursor for bioplastics (Niederholtmeyer *et al.*, 2010, Angermayr *et al.*, 2012). Several other products and strategies for their production are summarized in reviews (Angermayr *et al.*, 2015, Dexter *et al.*, 2015, Lai & Lan, 2015, Sarsekeyeva *et al.*, 2015, Savakis & Hellingwerf, 2015, Zhou *et al.*, 2016, Knoot *et al.*, 2018). The most famous and well characterized compound produced heterologously in cyanobacteria is ethanol, which is a biofuel already used abundantly in practice.

The production of ethanol by fermentation of pyruvate with the enzymes pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) of yeasts like *Saccharomyces cerevisiae* is probably the oldest application of biotechnology in human history (Figure 2).

PDC catalyses the irreversible decarboxylation of pyruvate to acetaldehyde and CO_2. ADH reduces acetaldehyde to ethanol with reducing equivalents from NADH. In bacteria PDC of bacterial origin is used for recombinant expression, most frequently of the origin of *Zymomonas mobilis* and often in combination with the respective ADH (Ingram *et al.*, 1987).

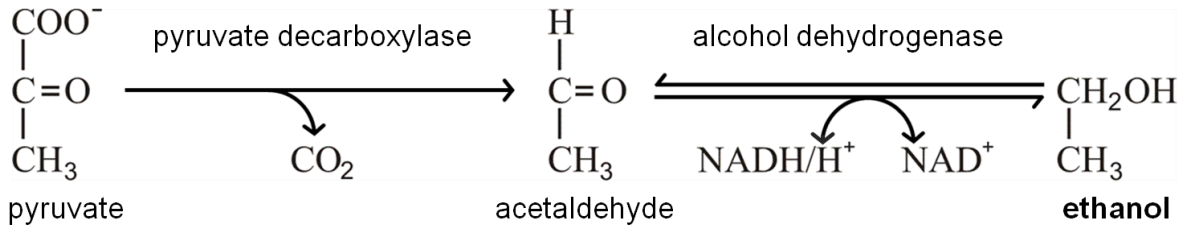


Figure 2: Production of ethanol from pyruvate. The reaction from ethanol to pyruvate is catalyzed by the enzymes pyruvate decarboxylase and alcohol dehydrogenase.

The recombinant production of ethanol was first achieved in *Synechococcus elongatus* PCC 7942 (Deng & Coleman, 1999). Production rates of ethanol were increased by different strategies since then: The use of strong promoters like the one of *psbAII* (photosystem II D1 protein) in *Synechocystis* sp. PCC 6803 (Dexter & Fu, 2009). The integration of the *pdc*/*adh* cassette at two integration sites into the genome and additional deletion of competing pathways like the PHB synthesis (Gao *et al.*, 2012). The optimization of the CO_2 fixation rate with the overexpression of four enzymes of the Calvin-Benson-Bassham cycle (Liang *et al.*, 2018). The use of NADPH dependent ADH, like the endogenous ADH (*slr1192*) of *Synechocystis* sp. PCC 6803 (Gao *et al.*, 2012, Dienst *et al.*, 2014), is a further optimization possibility in respect to the higher availability of NADPH compared to NADH in cyanobacterial cells (Park & Choi, 2017). Also the recombinant coexpression of a transhydrogenase, which catalyzes the electron transfer between NAD(H) and NADP(H) and vice versa, increased the production of lactic acid in cyanobacteria (Niederholtmeyer *et al.*, 2010, Angermayr *et al.*, 2012). This should be transferable to optimized ethanol production as well. High ethanol production can be achieved by *pdc*/*adh* cassette encoded on a plasmid (Dienst *et al.*, 2014) or integrated into the genome (Dexter & Fu, 2009, Gao *et al.*, 2012). Recombinant biotechnology in cyanobacteria is not only in the focus of academic research but there are also commercial companies active in the field like Algenol (USA), Photanol (the Netherlands), and Cyano Biotech (Germany).

1.2 Cyanobacteria

1.2.1 Ecological and geological food print of cyanobacteria and phylogeny

Cyanobacteria are the inventor of oxygenic photosynthesis and probably the organism group that changed the face of earth more significantly than any other. The time between 3.8 – 2.2 billion years ago is discussed as time of origin of the first cyanobacteria. The time range starts with the possibly first geological proof of photosynthetic life and ends with the great oxygenation event, when the atmospheric level of oxygen started to rise (Schopf, 2014). The oldest fossils for photosynthetic bacteria are more likely to rely on anoxygenic photosynthesis, which evolved before oxygenic photosynthesis (Schopf, 2014, Shestakov & Karbysheva, 2017). It is hypothesized that cyanobacteria originated distinctly before the great oxygenation event. The generated oxygen probably oxidized first S, H_2S, CH_4, and Fe^{2+} before it accumulated in the atmosphere, which explains the delay in rise of

atmospheric oxygen (Sessions *et al.*, 2009, Schopf, 2014). With the release of oxygen the atmosphere changed from an anoxic environment over ages towards present conditions, a requirement for complex life on earth. Furthermore, photosynthetic organisms are the predominant carbon source for heterotrophic life forms. Photosynthesis in eukaryotic organism has originated from the ancestral endosymbiosis of a cyanobacterium, either directly by primary endosymbiosis or by secondary or tertiary endosymbiosis of a photosynthetically active eukaryote (Keeling, 2010). Today, cyanobacteria are still important primary producers in the oceans. Contributions of 10% (Mangan *et al.*, 2016) to 25% (Flombaum *et al.*, 2013) to global primary production are discussed. Many cyanobacteria are also capable of fixing atmospheric nitrogen and play thus an important role in the availability of nitrogen in various ecosystems (see also 1.2.2).

Cyanobacteria are ubiquitous and representatives can be thus found not only in aqueous environments (fresh and sea water) but also in soil, on solid surfaces like rocks, and even in extreme environments as thermal springs, arid environments, and endolithic ecosystems (Garcia-Pichel *et al.*, 2001, Wierzchos *et al.*, 2006, Dadheech *et al.*, 2013). Some cyanobacteria build symbioses with other organisms like fungi (i.e. lichen), plants (for example nitrogen supply in bryophytes), protists as diatoms, and animals as sponges and corals (Hyvarinen *et al.*, 2002, Adams & Duggan, 2008, Venn *et al.*, 2008, Amin *et al.*, 2012).

Since their first description as “blue-green algae” the taxonomic classification of cyanobacteria changed several times. A still today often used classification from 1979 is considering cellular organization, cell division, and cell differentiation. Cyanobacteria are divided into five subsections: Subsection I and II include unicellular cyanobacteria that live as single cells or in colonial aggregates. It is differentiated between cells that reproduce by binary fission or budding (subsection I) or by multiple fission (subsection II). The subsections III, IV, and V include filamentous cyanobacteria. Members of subsection III do not show any cell differentiation, while members of subsection IV and V can differentiate from vegetative cells into other cell types like heterocysts. Members of subsection V can additionally build branching trichomes by division in more than one plane in contrast to subsection IV (Rippka *et al.*, 1979). The classification of cyanobacteria into subsections is still used today for indication of cell morphology or cell division but it is not relevant anymore in terms of relationship due to progress in electron microscopy and molecular genetics. For the indication of complexity however, this classification is still helpful. According to published literature, the classification of subsection IV and V (Tomitani *et al.*, 2006) or subsection V alone (Schirrmeister *et al.*, 2013) are consistent with present cyanobacterial phylogeny. Modern phylogenetic trees are constructed based on the molecular data of a number of conserved marker genes that allow higher resolution than trees only considering one gene like 16S rRNA (Komarek *et al.*, 2014, Walter *et al.*, 2017). An exemplary tree is shown in Figure 3.

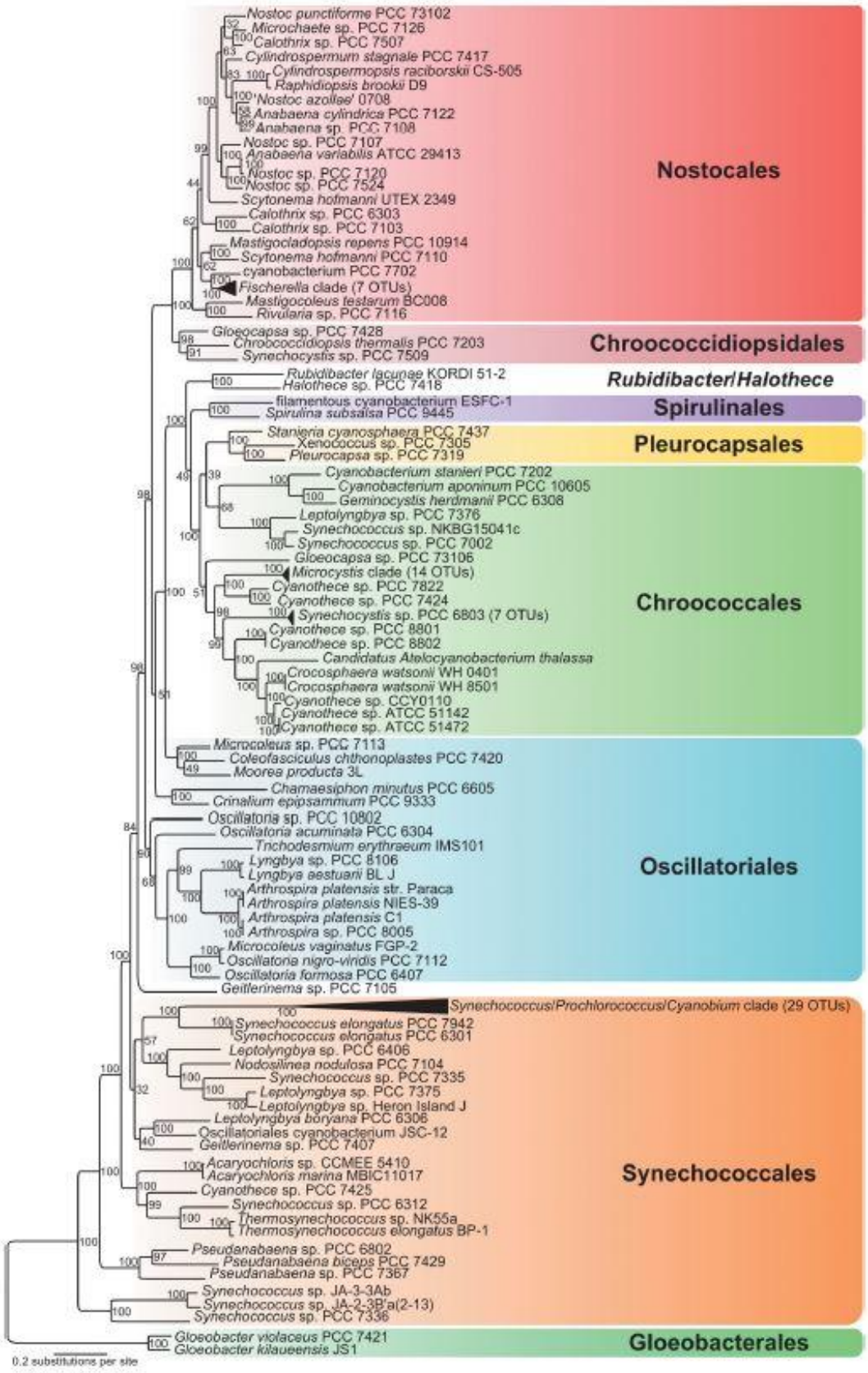


Figure 3: Phylogenetic tree of cyanobacteria from Komárek et al. 2014. This tree is based on an analysis with the maximum likelihood algorithm (one thousand bootstraps) of 31 conserved protein sequences of suitable genomes available in April 2014 (Komarek *et al.*, 2014).

1.2.2 Physiology of cyanobacteria

Cyanobacteria are gram negative and possess an outer membrane. Their cell wall also shows characteristics of a gram positive cell wall, such as high thickness for some cyanobacteria or a high cross-linking (Hoiczyk & Hansel, 2000). Cyanobacterial cells or filaments are surrounded by different polysaccharide layers named capsule, sheath, and mucilage. The sheath is thin and dense layer on the cell surface and usually visible by light microscopy (De Philippis & Vincenzini, 1998). Capsule and mucilage consist both of slimy material. While the capsule is still associated with the cell surface, the mucilage is dispersed into the surrounding environment (De Philippis & Vincenzini, 1998).

Light reaction of photosynthesis takes place in intracellular membrane systems, the thylakoids, where photosystem I and II are located. Most cyanobacteria grow photoautotrophically but also mixotrophic and heterotrophic cyanobacteria exist. *Gloeobacter* is the only genus known without thylakoids and photosynthesis most likely takes place in the cytoplasmic membrane (Rippka *et al.*, 1974). *Gloeobacter* is the first organism branching from the phylogenetic tree of cyanobacteria, even before the endocytosis of plastids happened and it is considered an ancient type of cyanobacterium (Criscuolo & Gribaldo, 2011). Nearly all cyanobacteria possess exclusively chlorophyll a and characteristic light-harvesting systems, the phycobilisomes. An exception are the genera *Prochlorothrix*, *Prochloron*, and *Prochlorococcus* that also possess chlorophyll b but no phycobilisomes (Bullerjahn & Post, 1993). There is also an example for chlorophyll d with *Acaryochloris marina* (Miyashita *et al.*, 1996). Phycobilisomes are soluble multi-protein complexes of phycobiliproteins that are anchored in the thylakoid membrane and associated with the photosystems (Grossman *et al.*, 1993, Watanabe *et al.*, 2014). The core of a phycobilisome is constituted of allophycocyanin, to which other phycobiliproteins are attached. The most common phycobiliproteins in cyanobacteria besides allophycocyanin are phycocyanin and phycoerythrin (Gantt, 1981). The chromophores of the three mayor phycobiliproteins are linear tetrapyrroles that are covalently attached to the respective protein (Grossman *et al.*, 1993), namely phycocyanobilin (for allophycocyanin and phycocyanin) and phycoerythrobilin (for phycoerythrin). Two additional chromophores are known with phycourobilin, which is an additional chromophore for certain types of phycocyanin and phycoerythrin in marine cyanobacteria, and phycoviolobilin, which is a chromophore in phycoerthrocyanin, a phycobiliprotein present in some cyanobacteria (MacColl, 1998, Blot *et al.*, 2009). The composition of phycobiliproteins in the phycobilisomes differs between different cyanobacteria and can also be adapted to the wavelength of a light source, which is called chromatic adaptation (Grossman *et al.*, 1993).

Cyanobacteria have several intracellular storage depots that can be detected as granules by electron microscopy. Phosphate can be stored in polyphosphate out of ATP by the enzyme polyphosphate kinase (Kromkamp, 1987). Nitrogen can be stored in the cyanobacterial specific storage compound cyanophycin, a polymer of the amino acids aspartic acid and arginine in a molar ratio of 1:1 (Kromkamp, 1987). The polymer consists of a polypeptide backbone of aspartic acid, which is

coupled to an arginine via a peptide bound of the beta-carboxyl group to the alpha-amino group of arginine. The reaction is catalyzed by the enzyme cyanophycin synthetase under ATP hydrolysis (Ziegler *et al.*, 1998). Glycogen is used as major carbon and energy storage in all cyanobacteria but many cyanobacteria can also store carbon as PHB (Troschl *et al.*, 2017).

Cyanobacteria as primary producers are not only an important source of organic carbon but many cyanobacteria are also diazotrophic organisms, i.e. possess the ability to fix atmospheric nitrogen. Cyanobacteria are thus an important source of biologically available nitrogen in ecosystems. The reaction of N_2 to ammonium is catalyzed by the enzyme nitrogenase. Nitrogenase is a multi-protein complex consisting of the heterotetramer dinitrogenase and of the homodimer dinitrogenase reductase (Peters & Szilagyi, 2006). Fixation of atmospheric nitrogen is very energy intensive: For one molecule of N_2 eight molecules NADH or NADPH and 16 molecules ATP are required (Tsygankov, 2007). Nitrogenase is vulnerable towards oxygen and reactive oxygen species and probably originated under low atmospheric oxygen conditions before the great oxygenation event (Berman-Frank *et al.*, 2003). Nitrogenase activity in cyanobacteria is challenging due to the oxygen generation through photosynthesis and the energy demands for N_2 fixation. Two general strategies emerged to avoid the inactivation of the nitrogenase by oxygen: Spatial separation is typical for heterocyst forming cyanobacteria while temporal separation is achieved by different time points of N_2 fixation and photosynthesis (Berman-Frank *et al.*, 2003). Cyanobacteria of the subsection IV and V are able to differentiate vegetative cells into heterocysts under nitrogen starvation. This N_2 fixing cell type is no longer photosynthetically active, its gas exchange with the environment is reduced by an extracellular glycolipid layer, and incoming oxygen is consumed by respiration (Flores & Herrero, 2010). Heterocysts and vegetative cells exchange reduced carbon metabolites against fixed nitrogen in form of amino acids (Kumar *et al.*, 2010). Temporal separation can be achieved by day/night cycle dependent and by cell-cycle dependent activity of these two mutual exclusive processes (Mitsui *et al.*, 1986, Stal & Krumbein, 1987). A unique strategy is described for the genus *Trichodesmium*, the probably most important N_2 fixing organism in the oceans (Langlois *et al.*, 2008, Grosskopf *et al.*, 2012). *Trichodesmium* cells can reversibly differentiate in diazocytes, in which oxygen evolution is reduced and N_2 fixation can take place in the light (Bergman *et al.*, 2013).

The number of chromosomes per cells differs among cyanobacterial species and strains. Although there are examples for species, which are with one chromosome per cell monoploid, most cyanobacteria have more than one chromosome copy and are considered – depending on the copy number – oligoploid or polyploid (Griese *et al.*, 2011). The regulation and the level of chromosome copy number are highly variable between different cyanobacterial species. For marine *Synechococcus* sp. WH7803 chromosome copy number is around four for various cultivation conditions (Perez-Sepulveda *et al.*, 2018). For *Synechococcus elongatus* PCC 7942 chromosome copy was demonstrated to be positively linear correlated with cell length (Chen *et al.*, 2012, Zheng & O'Shea, 2017). Vegetative cells of the Nostocales species *Aphanizomenon ovalisporum* have multiple chromosome

copies and in their dormant state (akinetes, see below) copy number is 15-fold increased (Sukenik *et al.*, 2012). Possible reasons for difference in chromosome copy number are given: It may guarantee the integrity of the genome during longer term of inactivity. It may also be a strategy for fast cell propagation after break of dormancy, since DNA replication is an energy-, resource-, and time-intensive process. A possible support for this hypothesis is that akinetes of *Aphanizomenon ovalisporum* do not contain polyphosphate granules and available phosphate is thus integrated into DNA (Sukenik *et al.*, 2012). There are more examples that DNA replication and cell division are decoupled in cyanobacteria. DNA replication machinery has higher activity during lag phase before exponential growth for *Synechococcus elongatus* PCC 7942 (Watanabe *et al.*, 2015, Murray, 2016). This finding fits the observation that the chromosome copy number of polyploid *Synechocystis* sp. PCC 6803 is growth rate dependent and decreases for later growth phases (Griese *et al.*, 2011, Zerulla *et al.*, 2016). A possible advantage of oligo- or polyploid lifestyle might be, besides lower requirements for fast propagation under favorable conditions (see above), that DNA damage can be repaired by homologous recombination (Perez-Sepulveda *et al.*, 2018). Several cyanobacteria were proven to show remarkably high resistance against radiation, which is in some cases comparable to *Deinococcus radiodurans*; an organism famous for its radiation resistance that is related to its multiple chromosome copies (Soppa, 2014, Cassier-Chauvat *et al.*, 2016).

1.2.3 Filamentous cyanobacteria

The filamentous growth of cyanobacteria is not a criterion in modern taxonomy based on molecular genetics. Filamentous growth can be found for some members of the Synechococcales, for most members of the Oscillatoriales and for all members of the Spirulinales and Nostocales (Komarek *et al.*, 2014). Possible advantages of filamentous lifestyle are defense against predators, reduced interaction with non-cooperative competitors, and the possibility of cell differentiation for instance (Herrero *et al.*, 2016). Filamentous cyanobacteria can be classified into a non-heterocyst and a heterocyst forming group. Differentiation into heterocysts is exclusive for members of the Nostocales. Vegetative cells of several members of the Nostocales can also differentiate in two other forms, hormogonia and akinetes (Flores & Herrero, 2010). Hormogonia are short, motile filaments that facilitate the colonization of new habitats and enable symbiosis with plants for some species (Meeks & Elhai, 2002). Akinetes are spore-like, dormant cells to outlast unfavorable, environmental conditions (Adams & Carr, 1981). There is evidence for the presence of a continuous periplasm for all three subsections of filamentous cyanobacteria (Herrero *et al.*, 2016). Members of the Nostocales show furthermore septal junctions that are allow diffusion driven, cytoplasmic exchange between different cells in a filament (Nieves-Morion *et al.*, 2017).

Many filamentous cyanobacteria are able to move along surfaces by gliding motility, but the underlying mechanism is not understood by now. Gliding motility is often correlated with slime

secretion and it was proposed that the secretion of polysaccharide slime by junctional pore complexes provides propulsion force (Hoiczyk & Baumeister, 1998). In contrast, recent findings indicate that type IV pili are essential for gliding motility of hormogonia of *Nostoc punctiforme* and that polysaccharide excretion is supportive but not essential for motility (Khayatan *et al.*, 2015, Cho *et al.*, 2017). Thus, the driving force of movement probably relies on the type IV pili similar to the twitching motility of unicellular cyanobacteria (Khayatan *et al.*, 2015, Wilde & Mullineaux, 2015).

1.2.4 *Phormidium lacuna*

The taxonomy of the genus *Phormidium* is still under investigation. This genus of the order Oscillatoriales is widespread and considered polyphyletic (Marquardt & Palinska, 2007, Komarek *et al.*, 2014). On the taxonomic level of family, the classification differs among *Phormidium* species and they are distributed among the Phormidiaceae, Oscillatoriaceae, and Microcoleaceae (Sciuto *et al.*, 2012, Komarek *et al.*, 2014). The concept of the Phormidiaceae in its original form is rejected by some authors (Strunecky *et al.*, 2014), but this family name is still used in more recent publications (da Silva Malone *et al.*, 2015, Martins & Branco, 2016).

The organism used in this study is characterized as member of the genus *Phormidium* according to 16S rRNA and core proteome based phylogenetic analyses (Nies *et al.*, 2017). It was furthermore characterized as new species denominated *Phormidium lacuna*. Based on the core proteome analysis *Phormidium lacuna* and its closest relatives in the genus *Phormidium* are grouped with *Geitlerinema* sp. PCC 7105 (Figure 3). Several strains of *Phormidium lacuna* were isolated by members of this workgroup from marine rockpools in the North Sea (Helgoland, Germany) and the Mediterranean Sea (Giglio, Italy). Rockpools (or tidal pools) are in close proximity to the sea without direct contact. Environmental conditions like temperature or salt concentration can have a broad spectrum due to invading sea water, evaporation, and rain fall. *Phormidium lacuna* was characterized as fast propagating, capable of N_2 fixation, and tolerant to different salt concentrations and to temperatures up to 50 °C (Nies *et al.*, 2017). *Phormidium lacuna* was considered a promising candidate for cyanobacterial biotechnology and it was demonstrated to be generally cultivatable in bioreactors (Nies *et al.*, 2017). In suspension culture, *Phormidium lacuna* shows floating and adherent growth, while floating growth dominates under standard cultivation condition. *Phormidium lacuna* filaments show strong tendency to aggregate in suspension culture.

1.3 Gene transfer into cyanobacteria

There are three common methods for gene transfer into cyanobacteria: Natural transformation, conjugation, and electroporation. Natural transformation differs from the other two methods since the recipient cell actively transports DNA into the cytoplasm. In literature reviews natural transformation is often described as a rare trait among cyanobacteria exclusive for a small group of unicellular species (Koksharova & Wolk, 2002, Vioque, 2007, Al-Haj *et al.*, 2016). In fact, natural transformation is

described for several strains of *Synechococcus* (Shestakov & Khyen, 1970, Stevens & Porter, 1980) and *Synechocystis* (Devilly & Houghton, 1977, Grigorieva & Shestakov, 1982), and there are also other examples with *Microcystis aeruginosa* PCC 7806 (Dittmann *et al.*, 1997), and *Thermosynechococcus elongatus* BP-1 (Onai *et al.*, 2004), which are all unicellular. But additionally there is also one example of natural transformation for a filamentous cyanobacterium with *Nostoc muscorum* (Trehan & Sinha, 1982). In contrast to the unicellular cyanobacteria, for *Nostoc muscorum* only mutant complementation but no heterologous gene expression was demonstrated. This may be a reason why this representative is not considered in many reviews on natural transformation in cyanobacteria.

Conjugation relies on conjugative plasmids that allow interspecies DNA transfer between a donor and a recipient cell. Conjugation is a coupled process of rolling circle replication and DNA transport into the recipient cell by the type IV secretion system after mating pair formation. Conjugation is initiated by specific site on the plasmid, the origin of transfer, while the respective genes for conjugation can be encoded on the transferred plasmid but also on another plasmid or on the chromosome (Llosa *et al.*, 2002, Cabezon *et al.*, 2015, Wawrzyniak *et al.*, 2017). Gene transfer via conjugation from an *Escherichia coli* donor strain into cyanobacteria was described first for members of the Nostocales and *Synechococcus* (Wolk *et al.*, 1984). There are also further examples for gene transfer via conjugation for all subsections of the cyanobacteria (Koksharova & Wolk, 2002), for instance for *Synechocystis*, *Chroococcidiopsis*, and *Fischerella* (Kreps *et al.*, 1990, Billi *et al.*, 2001, Stucken *et al.*, 2012).

Electroporation is a universal method for gene transfer in all domains of life. By electroporation small pores are introduced temporarily in the cell membrane, which is thus permeabilized for water soluble molecules during a short-time period (Neumann *et al.*, 1982, Sukharev *et al.*, 1992). For cyanobacteria electroporation was first described for the genus *Anabaena* (Thiel & Poo, 1989). Today, there are also examples for all subsections of the cyanobacteria to be transformable by electroporation like *Synechococcus*, *Synechocystis*, *Microcystis*, *Chroococcidiopsis*, *Nostoc*, and *Fischerella* (Matsunaga *et al.*, 1990, Moser *et al.*, 1993, Tillett *et al.*, 2000, Billi *et al.*, 2001, Ludwig *et al.*, 2008, Stucken *et al.*, 2012) and in particular there are examples for several members of the order Oscillatoriales like *Leptolyngbya*, *Arthrospira*, *Oscillatoria*, and *Planktothrix* (Fujita *et al.*, 1992, Toyomizu *et al.*, 2001, Ravindran *et al.*, 2006, Ishida *et al.*, 2007). In Table 1 all genera of cyanobacteria capable of natural transformation are listed as well as selections of the genera, in which gene transfer with conjugation or electroporation was demonstrated.

Table 1: Methods for gene transfer in a selection of cyanobacteria. All natural transformable genera are shown as well as a selection of cyanobacteria, in which gene transfer by conjugation or electroporation was demonstrated. For the genus *Nostoc* only mutant complementation was shown for one species but not heterologous gene expression (grey characters).

Method	Order	Genera	
Natural transformation	Synechococcales	*Synechocystis, Synechococcus, Thermosynechococcus*	u
	Chroococcales	*Microcystis*	u
	Nostocales	*Nostoc*, only mutant complementation	f
Conjugation	Synechococcales	*Synechocystis, Synechococcus*	u
	Oscillatoriales	*Leptolyngbya*	
	Chroococcidiopsidales	*Chroococcidiopsis*	u
	Nostocales	*Anabaena, Nostoc, Fischerella*	f
Electroporation	Synechococcales	*Synechocystis, Synechococcus*	u
	Oscillatoriales	*Oscillatoria, Leptolyngbya, Arthrospira, Planktothrix*	f
	Chroococcales	*Microcystis*	u
	Chroococcidiopsidales	*Chroococcidiopsis*	u
	Nostocales	*Anabaena, Nostoc, Fischerella*	f
(u - unicellular; f - filamentous)			

1.3.1 Mechanism of natural transformation

Natural transformation (NT) in bacteria was first described by Griffith in 1928 as "transformation of type". He observed that the non-virulent R form of *Streptococcus pneumoniae* can be transformed to the virulent S form if R form cultures are inoculated with killed S form cultures (Griffith, 1928). This experiment is the basis for later work of Avery and coworkers that first proved that DNA is the carrier of genetic information. They demonstrated that the R form can only be transformed by the DNA of the S form (Avery *et al.*, 1944). The role of DNA as material encoding genetic information was accepted finally by the demonstration that during bacteriophage infection of *Escherichia coli* only DNA but no proteins enter the host cell (Hershey & Chase, 1952).

Natural competence in bacteria has been studied in several gram positive and gram negative model organisms, which are often human pathogens. Natural competence describes the physiological state of a prokaryote, in which it is able to actively take up DNA from the environment. Natural competence is often monitored by natural transformation, the active uptake from environmental DNA and its integration into the genome. Hence, both terms, natural competence and natural transformation, are often used as synonyms because they are not monitored separately and natural competence is a prerequisite of NT.

Natural competence in bacteria relies on the type IV pilus related structures, even though one exception is known with *Helicobacter pylori* (Stingl *et al.*, 2010). The denomination of the proteins involved in NT differs in some cases between the studied organisms. The following description of NT is based on a recent review of natural competence in the gram negative model organism and human pathogen *Vibrio cholerae* (Matthey & Blokesch, 2016) if not stated otherwise. The proteins of the Type IV pilus, the type IV pilins, are present in bacteria and archaea. They are involved in different

arrangements in diverse function as motility, adhesion, protein secretion, and DNA uptake (Berry & Pelicic, 2015). The working model of NT in *Vibrio cholerae* (see below and Figure 4) is based on experimental data in this organism and other bacteria but also on assumptions, since not all steps were confirmed experimentally (Matthey & Blokesch, 2016).

The major pilin PilA is the subunit of the main part of the pilus. Before PilA can be incorporated in the pilus it is processed by the prepilin peptidase PilD. The extension of the pilus is facilitated by PilB and the retraction by PilT in an ATP dependent manner. The type IV pilus is anchored in the inner membrane and it crosses the outer membrane through a pore formed by PilQ subunits. Further parts of the type IV pilus are PilC, PilM, PilN, PilO, PilP, and PilF. PilF is required for the localization and multimerization of PilQ (Koo *et al.*, 2008). The pilins PilC, PilM, PilN, PilO, and PilP are important for the pilus assembly and may facilitate interaction with the cytosolic ATPases PilB and PilT and other parts of the pilus (Takhar *et al.*, 2013, Tammam *et al.*, 2013).

It is postulated that parts of the pilus bind to DNA and it is moved into the periplasma by retraction of the pilus. Even though a functional type IV pilus significantly increases transformation efficiency, several mutants lacking parts of the type IV pilus of *Vibrio cholerae* are still transformable. In contrast, the competence proteins required for the transport across the inner membrane, ComEA, ComEC, and ComF, seem to be mandatory for natural competence. In the periplasm double stranded DNA is bound by ComEA and single stranded DNA enters the cytoplasm through an inner membrane channel of ComEC subunits. It is hypothesized that once the DNA is bound by ComEA in the periplasm it cannot move back through the PilQ pore but only further into the periplasm. DNA is thus pulled into the periplasm by the binding of increasing numbers of ComEA proteins. Also binding of ComEA compacts the DNA. How the double stranded DNA is degraded into a single strand and how DNA is translocated from ComEA to ComEC is still not known. Also the mechanism of transport across the inner membrane is still under investigation. The protein ComF is required for this process but its function is still unknown. Furthermore, the role of the helicase PriA is discussed. PriA is an ATP-dependent DNA helicase and might drive ComEC related DNA transport into the cytoplasm. Single stranded DNA entering the cytoplasm is bound by single strand binding proteins (Ssb) and DprA (DNA processing protein A) and thus protected against degradation. DprA furthermore facilitates the binding of RecA (Recombinase A) to the DNA. RecA proteins and DNA form a filament together with a stoichiometry of one RecA molecule every 3 bp (Dombroski *et al.*, 1983). This nucleoprotein filament scans double stranded DNA for homologous sequences by comparably stronger interaction with homologous than heterologous sequences (Forget & Kowalczykowski, 2012, Ragunathan *et al.*, 2012). The screening of homologous sequences by the nucleoprotein filament is a prerequisite step for homologous recombination. Homologous recombination is a conserved mechanism, which enables genome stability and recombination and numerous reviews exist in literature (Persky & Lovett, 2008, Bell & Kowalczykowski, 2016).

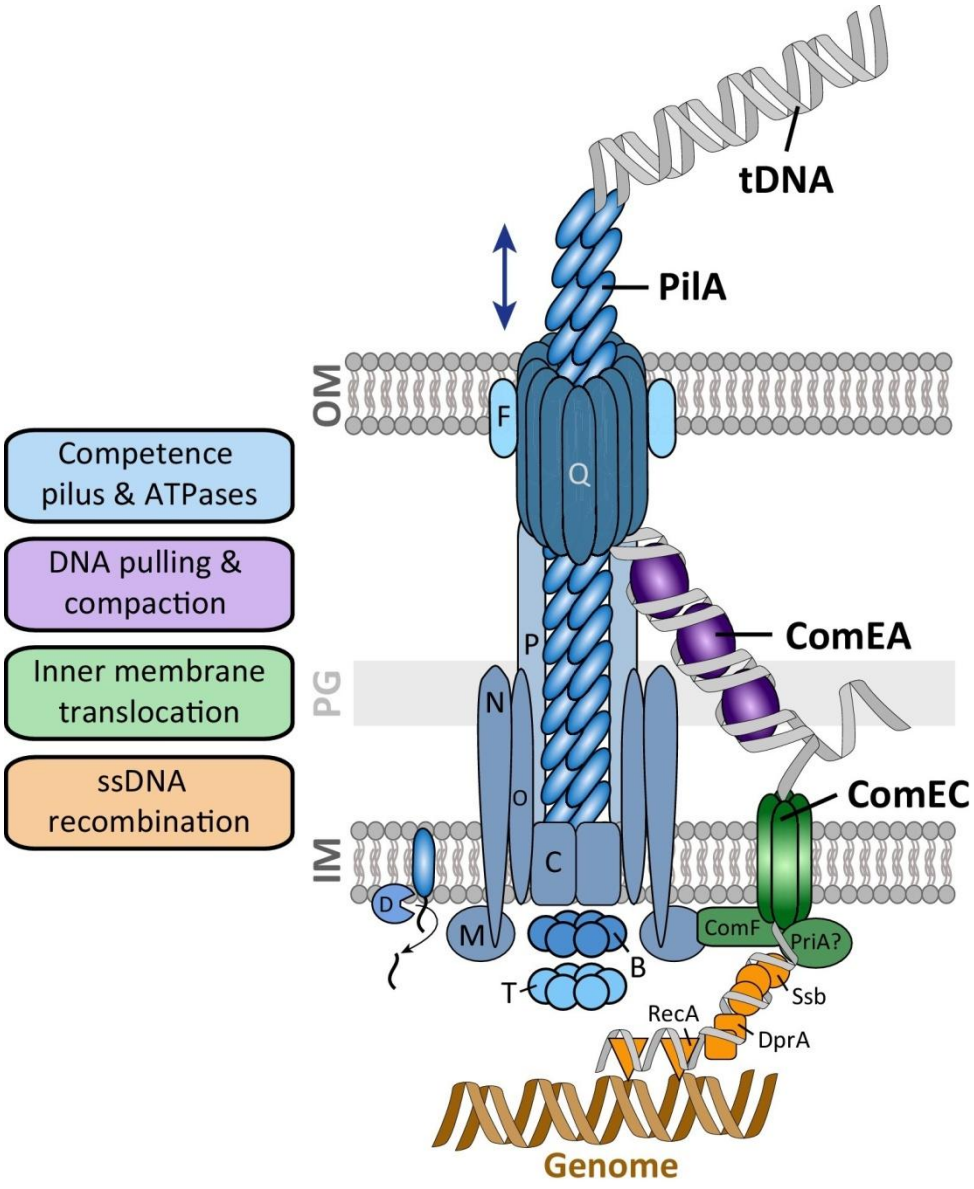


Figure 4: Molecular model of natural transformation in the gram negative bacterium *Vibrio cholerae* from Matthey and Blokesch, 2016. Involved proteins are divided into the type IV pilus (blue), ComEA (purple), proteins involved into the inner membrane translocation of DNA (green), and proteins involved in binding of single stranded DNA and recombination (orange). Pilins names beside PilA are abbreviated to single letters. Main part of the pilus, PilA, is processed by the prepilin peptidase PilD. Pilus elongation and retraction (addition or subtraction of PilA subunits) is facilitated by the ATPases PilB and PilT, respectively. Transforming DNA (tDNA) is transported into the periplasm through the PilQ pore across the outer membrane (OM) by pilus retraction and bound by ComEA. Further transport into the cytoplasm is supported by binding of further ComEA proteins. ComF and probably PriA are involved in the DNA transport across the inner membrane (IM) through the ComEC channel. DNA reaching the cytoplasm is single stranded and bound subsequently by single strand binding proteins (Ssb) and DprA. RecA is recruited by DprA and enables recombination of tDNA into the genome. For further information consider text. PG: Peptidoglycan (Matthey & Blokesch, 2016).

Several mutants for proteins involved in NT were generated for *Synechocystis* sp. PCC 6803. It was reported that mutants for *comEA*, *comF*, *pilA1*, *pilB1*, *pilD*, *pilM*, *pilN*, *pilQ*, and *pilT1* (Bhaya *et al.*, 2000, Yoshihara *et al.*, 2001, Okamoto & Ohmori, 2002, Nakasugi *et al.*, 2006) are deficient in NT.

Together with *pilC* mutants (Bhaya *et al.*, 2000) the mutants of the pilins above furthermore show lack in motility and changed structure of the thick pili of *Synechocystis* sp. PCC 6803. *Synechocystis* sp. PCC 6803 also has thin pili that facilitate adherence to other cells or other surfaces and that do not play a role in motility or natural competence (Schuergers & Wilde, 2015). *pilF* and *pilP* are absent in *Synechocystis* sp. PCC 6803 (Schuergers & Wilde, 2015). The respective mutants demonstrate that natural competence in *Synechocystis* sp. PCC 6803 is linked to functional type IV pili.

1.4. Scope of this work

The central point of this work was to increase the potential of cyanobacterial biotechnology by the introduction of a potentially promising organism to the field. *Phormidium lacuna* was characterized recently by our workgroup as a robust and fast-growing organism (Nies *et al.*, 2017). The main objective of this study was to make *Phormidium lacuna* accessible for recombinant biotechnology and to characterize its potential for biotechnology by the recombinant production of ethanol. So far no transformation protocol was available for the genus *Phormidium*. Gene transfer via electroporation is described for several other genera of the order Oscillatoriales while there is only report of conjugation with the genus *Leptolyngbya* and no example of natural transformation in this order (Table 1). Therefore, electroporation was the method of choice to establish gene transfer into *Phormidium lacuna*. During the establishment of the transformation protocol, it was revealed that efficient gene transfer via natural transformation is possible into *Phormidium lacuna*.

A reliable and efficient transformation protocol was a prerequisite to enable recombinant ethanol production in *Phormidium lacuna*. The genes encoding for pyruvate decarboxylase and alcohol dehydrogenase were integrated into the genome of *Phormidium lacuna*. Ethanol production of the respective transformants was monitored under different cultivation conditions.

Natural transformation allows efficient and reliable gene transfer into an organism and is thus a preferable method for gene transfer. However, there are only few examples for natural transformation among cyanobacteria so far. Therefore, it is a relevant question if the findings of natural transformation among cyanobacteria are isolated events or if natural competence is a more common trait in this phylum. In the second case more cyanobacteria would be accessible for recombinant biotechnology, what could increase its potential significantly. To investigate the distribution of natural competence among cyanobacteria a bioinformatic analysis was performed. The sequences of proteins, which facilitate natural competence in cyanobacteria, were compared with the total protein sequences of representatives of the cyanobacterial phylum. Promising candidates to test for natural transformation were predicted based on sequence homology with naturally competent cyanobacteria.

2 Material and Methods

Chemicals were ordered from Carl Roth (Germany) and level of purity was > 99% (or higher) if not stated otherwise. Ultrapure water (Purelab flex, ELGA LabWater, UK) was used for all solutions and media if not stated otherwise. For microbiology work all solutions and media were sterilized by autoclaving or sterile filtration before use and all working steps were performed under clean bench.

2.1 Molecular biology

All steps in molecular biology were performed with reagents from NEB (USA) according to the manufacturer's instructions if not stated otherwise. Nuclease free water (Lonza, Switzerland) was used in molecular biology.

2.1.1 Polymerase chain reaction (PCR)

Preparative PCRs were performed with Q5 polymerase, analytic PCRs with Taq polymerase following the manufacturer's instructions. Standard PCR mixture and program are listed is Table 2. PCR programs were adapted for the respective requirements if necessary.

Table 2: Standard protocols for PCR reaction with Taq or Q5 polymerase (NEB). Reaction volumes of 50 µl are indicated. Depending on the experiment 50 µl, 25 µl, or 12.5 µl total volume reactions were used. Annealing temperature was adjusted to the primers binding sites. Elongation time was adjusted to the expected PCR product size. Standard protocols were optimized depending on the specific experimental conditions if necessary. One unit of a polymerase is defined by the manufacturer as the amount of enzyme that will incorporate 10 nmol of dNTP into acid insoluble material in 30 minutes (Taq: 37 °C; Q5: 74 °C).

	Taq polymerase	Q5 polymerase	concentrations, remarks
PCR mixture			
reaction buffer	5 µl	10 µl	
dNTPs	1 µl	1 µl	10 mM
forward primer	1 µl	1.5 µl – 2.5 µl	10 µM
reverse primer	1 µl	1.5 µl – 2.5 µl	10 µM
DNA template	variable	variable	
polymerase	0.1 – 0.25 µl	0.25 – 0.5 µl	Taq: 5 unit/µl, Q5: 2 unit/µl
nuclease-free water	to 50 µl	to 50 µl	
PCR program			
initial denaturation	95 °C, 30 s	98°C, 30 s	5 min for cell sample
denaturation	95 °C, 30 s	98°C, 10 s	
annealing	primer specific, 1 min	primer specific, 30 s	Q5: 40 s for cell sample
elongation	68 °C, 1 min/kb	72°C; 30 s/kb	30 – 35 cycles
final elongation	68 °C; 5 min	72 °C; 5 min	
storage	12 °C	12 °C	

As PCR templates either purified plasmid DNA or cell samples of *Phormidium lacuna*, *Synechocystis* sp. PCC 6803, or *Escherichia coli* DH5alpha were used. Initial denaturation step of PCR program was prolonged to 5 min for cell samples. *Phormidium lacuna* samples were homogenized with micropestle and optionally frozen over night before use as PCR template. PCR products were analyzed on agarose gels with SYBR safe (Invitrogen, USA). Remaining template (plasmid DNA) was removed by DpnI digestion. Products of preparative PCRs were purified with Invisorb Fragment Cleanup kit (Stratec molecular, Germany). All used primers were ordered from Sigma-Aldrich (USA) and are in Table 3.

Table 3: Primer for construction of plasmids and transformant validation. Forward primer: fwd. Reverse Primer: rev. Type II restriction sites: BmtI, XbaI. Type IIs restriction sites: BbsI, BsmBI.

Name	Purpose	Sequence
Primers for construction of plasmids		
t244	Amplification of 02_124, fwd	TGTCGTCATCTGTTTAGTCGTAAC
t245	Amplification of 02_124, rev	CGGTTCTTGCAAATCATGAGAGG
t252	Amplification of 55_26, fwd	ATGACAAACATTAACATCGGCTCC
t253	Amplification of 55_26, rev	TAACAGATCCGTGGTAACTGTTC
t256	Amplification of 07_37, fwd	CGTGCGAGACTCAACCCAAAC
t257	Amplification of 07_37, rev	GAAACCTGATCGAACCGTTTTAC
F114	Amplification of 07_37, fwd (long sequence)	TTGTTCGAGGCAGTTGCG
F115	Amplification of 07_37, rev (long sequence)	TGACAATGGGGTGGAGGG
F3	Construction of 55-K, vector fragment, fwd, BmtI	CAACAAGCTAGCCGAGACAGCTATTGAAAGCG
F4	Construction of 55-K, vector fragment, rev, XbaI	CAACAATCTAGAAACATTAACCCCTCGCGAAC
F5	Construction of 7-K or 7-K:2k, vector fragment, fwd, BmtI	CAACAAGCTAGCGTTTGCGAGGCTAAAGGCG
F6	Construction of 7-K or 7-K:2k, vector fragment, rev, XbaI	CAACAATCTAGAGGTTCCCACTCCCAAAGC
F7	Construction of 2-K, vector fragment, fwd, BmtI	CTTCTTGCTAGCTTGCGGGTAAAACCCCCTC
F8	Construction of 2-K, vector fragment, rev, XbaI	CAACAATCTAGAAATCGCAGACCCCATCGCG
F13	Construction of K plasmids, *kanR* operon, fwd, XbaI	CAACAATCTAGACTCGTATGTTGTGTGGAATTG
F14	Construction of K plasmids, *kanR* operon, rev, BmtI	CAACAAGCTAGCCAAGTCAGCGTAATGCTCTG
GG1	Construction of Kn plasmids, *kanR* operon, fwd, BbsI	CAACAAGAAGACGGAACCTAGGCACCCCAGGCTTTACAC
GG2	Construction of Kn plasmids, *kanR* operon, rev, BbsI	CAACAAGAAGACGCAAACTTTGCTTTGCCACGGAACGG
GG3	Construction of 7-Kn or 7-Kn:2k, vector fragment, fwd, BbsI	CAACAAGAAGACCCGTTTGCGAGGCTAAAGGC
GG4	Construction of 7-Kn or 7-Kn:2k, vector fragment, rev, BbsI	CAACAAGAAGACACGGTTCCCACTCCCAAAGC
GG5-1	Construction of 7-P-K:2k, 7-pP-A_K:2k, BsmBI	CAACAACGTCTCGTTATGAGTTATACTGTCGGTACCTATTTAGCG
GG6	Construction of 7-P-K:2k, BbsI	CAACAAGAAGACTCGACCTAGAGGAGCTTGTTAACAGGC
GG7	Construction of 7-P-K:2k, BbsI	CAACAAGAAGACTAGGTCGACTGAGGTTATAGCTATGAGCCATATTCAACGGGAAACG
GG8	Construction of 7-P-K:2k, 7-pP-A_K:2k, BbsI	CAACAAGAAGACTCATAACACCCCTTGTATTACTGTTTATGTAAGC

Table 3 (continued): Primer for cloning and transformant validation. Forward primer: fwd. Reverse Primer: rev. Type II restriction sites: BmtI, XbaI. Type IIs restriction sites: BbsI, BsmBI.

GG12-1	Construction of 7-P-A-K:2k, BsmBI	CAACAACGTCTCTCTAGGTCGACTGAGGTTATAGCTATGGC
GG13-1	Construction of 7-P-A-K:2k, 7-pP_K:2k, BsmBI	CAACAACGTCTCCCTAGAGGAGCTTGTTAACAGGC
GG14-1	Construction of 7-P-A-K:2k, BsmBI	CAACAACGTCTCCTTAGAAAGCGCTCAGGAAGAGTTC
GG15	Construction of 7-P-A-K:2k, BbsI	CAACAAGAAGACTTCTAAGTCGACTGAGGTTATAGCTATGAGC
GG16	Construction of 7-pP_K:2k, 7-pP-A_K:2k, BbsI	CAACAAGAAGACTTATGAGCCATATTCAACGGGAAAC
GG17	Construction of 7-pP_K:2k, 7-pP-A_K:2k, BbsI	CAACAAGAAGACGCTCATAACACCCCTTGTATTACTG
GG21	Construction of 7-pP_K:2k, BbsI	CAACAAGAAGACTGCTCGTATGTTGTGTGGAATTGTG
GG22	Construction of 7-pP_K:2k, BbsI	CAACAAGAAGACTACGAGCAAGTCAGCGTAATGCTCCG
GG23	Construction of 7-pP_K:2k, BbsI	CAACAAGAAGACCTCTAGGTTAACCCCAGCACCAACC
GG26	Construction of 7-pP-A_K:2k, BbsI	CAACAAGAAGACAAGTTAACCCCAGCACCAACC
GG27-1	Construction of 7-pP-A_K:2k, BsmBI	CAACAACGTCTCTTAACTTAGAAAGCGCTCAGGAAGAGTTC
F62	Construction of 7-GFP_Kn, NEBuilder, insert	CGCCTTTAGCCTCGCAAACGCTAGCTTTGCTTTGCCACGGAACG
F63	Construction of 7-GFP_Kn, NEBuilder, insert	AGTGTTCTTTAGGCACCCCAGGCTTTACAC
F64	Construction of 7-GFP_Kn, NEBuilder, insert	AAGCCTGGGGTGCCTAAAGAACACTCTAGGAATATGGC
F65	Construction of 7-GFP_Kn, NEBuilder, insert	GGACGAGCTGTACAAGTAATCTTAGCCCTTGGGAAGTTAGC
F66	Construction of 7-GFP_Kn, NEBuilder, insert	GGGCTAAGATTACTTGTACAGCTCGTCCATGC
F70	Construction of 7-GFP_Kn, NEBuilder, insert	AGCTTTGGGAGTGGGAACCTCTAGAGAGCTGTTTGAGCATCCC
F71	Construction of 7-GFP_Kn, NEBuilder, insert	CGCCCTTGCTCACCATTGAATTAATCTCCTACTTGACTTTATGAGTTGG
F72	Construction of 7-GFP_Kn, NEBuilder, insert	AGATTAATTCAATGGTGAGCAAGGGCGAG
F103	Removal of promoter from pGEMTeasy backbone	CAACAAAGATCTGTGAGCTAACTCACATTAATTGCG
F104	Removal of promoter from pGEMTeasy backbone	CAACAAAGATCTCCAAGCTATTTAGGTGACACTATAG
Primer for transformant validation		
F25	binding in genome (7-37) and on 7-[X]:2k, fwd	GGTCTAGGTGAGGCAATCC
F26	binding in KanR, rev	GTAATCATGGTCATAGCTGTTTCC
F27	binding in KanR, fwd	GCAGTTTCATTTGATGCTCG
F28	binding in genome (7-37) and on 7-[X]:2k, rev	ACCTGATTTGTTTATATCTGACGC
F29	binding in KanR sequence, fwd	CTATGACCATGATTACGAATTCCC
F30	binding in KanR sequence, rev	AAGCCGTTTCTGTAATGAAGG
F31	binding in AmpR sequence, fwd	GTTACCAATGCTTAATCAGTGAGG
F32	binding in AmpR sequence, rev	CGCTCATGAGACAATAACCC
F120	binding in genome (7-37), fwd	GGGTAGCCTAGACTCATCC
F121	binding in genome (7-37), rev	ATGCGGAAGTGACTGAGG
F123	binding in PDC sequence, rev	TCAGCAAATTTGACAGCAGC
F124	binding in ADH sequence, rev	AGAACTGCGGTAACAGTCG

2.1.2 Construction of plasmids

Vector maps for all generated plasmids, the respective sequence information, and the construction history are listed in the electronic supplement (see plasmids - maps and sequences). For an exemplary vector map also consider Figure 8. Plasmids for homologous recombination were based on the vector pGEM-T Easy (Promega, USA), which is supplied in linearized form and with 3' thymine overhangs. PCR products were integrated into pGEM-T Easy after A-tailing according the manufacturer's instructions. Sequences from three different loci on the *Phormidium lacuna* genome were used for homologous recombination, encoding for three proteins: phl#1409, phl#3443, and phl#3878 (further information under 3.1.1). Sequences were amplified via PCR with the respective primers (Table 3). Resulting homologous sequences in the pGEM backbone were ca. 1000 bp long and were named 02_124 (phl#1409), 55_26 (phl#3443), and 07_37 (phl#3878). For 07_37 also an ca. 2000 bp long homologous sequences was generated, abbreviated with the affix ":2k". The nucleotide sequence of the different loci can be found in the NCBI database under NZ_FSSI02000003.1 (02_124), NZ_FSSI02000056.1 (55_26), and NZ_FSSI02000008.1 (07_37) in combination with the respective primer sequences that define the used sequences (Table 3).

The kanamycin resistance operon (*kanR*), which encodes an aminoglycoside phosphotransferase, was used as a selection marker. The *kanR* operon originated from the plasmid pUC4K (Vieira & Messing, 1982). It was obtained from two sources. The first version of the *kanR* operon was cloned out of the plasmid pF10-His-KanR. In this plasmid of our workgroup, which originates from the plasmid pF10-His for the expression of a cyanobacterial phytochrome (Lamparter *et al.*, 1997), the open reading frame (ORF) is interrupted by the *kanR* operon. The sequence of the plasmid differed from the vector map information. The unexpected sequence led to a change in the 3' UTR (untranslated region) of the *kanR* operon. Later, the *kanR* operon was cloned out of the original source, pUC4K, with longer 5' and 3' sequences. The two different operons are designated KanR (first version) and KanRn (second version) and are abbreviated K and Kn for plasmid denomination. The *kanR* operon was integrated in the homologous sequences mentioned above with restriction enzyme based cloning according to manufacturer's instructions. Type II and Type IIs restriction enzymes were used. Restriction sites were inserted with the primers during PCR amplification of vector (see Table 3). DNA from restriction digest was purified in the same way like PCR products (see above), ligated with T4 DNA ligase, and transformed in chemically competent *Escherichia coli* DH5alpha with heat shock according to manufacturer's instructions. Plasmid DNA was purified from overnight culture (Roti-Prep – Plasmid MINI, Roth, Germany) and correct generation of plasmid was controlled by Sanger based sequencing (GATC, Germany). The resulting plasmids are listed in Table 4.

Plasmids encoding additionally PDC or PDC/ADH combined were generated the same way (PDC: pyruvate decarboxylase, ADH: alcohol dehydrogenase). Either *pdc* or *pdc*/*adh* were designed to be under the control of the same promoter as *kanR* (7-P-K:2k, 7-P-A-K:2k) or *kanR* and *pdc* or *pdc*/*adh* were under control of separate promoters, which both used the promoter sequence of the *kanR* operon

(7-pP_K:2k, 7-pP-A_K:2k; see also lower part of Table 4). The sequences of *pdc* and *adh* were derived from the sequence of *Zymomonas mobilis* and were obtained from plasmid pF10-His-KanR-petJ-PA. In this plasmid of our workgroup, which originates from the plasmid pF10-His-KanR (see above), the *petJ* promoter of *Synechocystis* sp. PCC 6803 (Kuchmina *et al.*, 2012) and the *pdc*/*adh* operon of *Zymomonas mobilis* DSMZ 473 (strain obtained from R. Geisen, Max Rubner Institute, Germany) were integrated. For plasmids with longer homologous sequences (:2k) and for 7-Kn, the promoter sequence on the pGEMTeasy backbone were partially removed with PCR and a subsequent one fragment cloning step because of homologies with the promoter of the *kanR* operon. Thereby, complications during the subsequent generation of plasmids were avoided.

Table 4: Plasmids used in this study for the transformation of *Phormidium lacuna*. Plasmids with bold letters were transformed successfully into *Phormidium lacuna*. The design principle of plasmids for homologous recombination in illustrated in the lower part of the table. Red: homologous sequences. Blue: *kanR* operon (promoter and coding sequence). Green: *pdc* or *gfp* coding sequence, Yellow: *adh* coding sequence, Grey: *cpcB* promoter for GFP expression. Sequence of the inserts and vector maps are listed in the electronic supplement (see plasmids - maps and sequences).

Plasmid name	Abbreviation	Locus	Genes
pGEMTeasy-02_124-KanR	2-K	phl#1409	*kanR*
pGEMTeasy-55_26-KanR	55-K	phl#3443	*kanR*
pGEMTeasy-07_37-KanR	7-K	phl#3878	*kanR*
pGEMTeasy-07_37-KanRn	7-Kn	phl#3878	*kanR*
pGEMTeasy-07_37-KanR:2k	7-K:2k	phl#3878	*kanR*
pGEMTeasy-07_37-KanRn:2k	7-Kn:2k	phl#3878	*kanR*
pGEMTeasy-07_37-PDC-KanR:2k	7-P-K:2k	phl#3878	*pdc*, *kanR*
pGEMTeasy-07_37-pPDC_KanR:2k	7-pP_K:2k	phl#3878	*pdc*, *kanR*
pGEMTeasy-07_37-PDC-ADH-KanR:2k	7-P-A-K:2k	phl#3878	*pdc*, *adh*, *kanR*
pGEMTeasy-07_37-pPDC-ADH_KanR:2k	7-pP-A_K:2k	phl#3878	*pdc*, *adh*, *kanR*
pGEMTeasy-07_37-pcpc(Syn)-GFP_KanRn	7-GFP_Kn	phl#3878	*gfp*, *kanR*
pVZ321	pZV321	-	*kanR*

Design principle of plasmids for homologous recombination

2-K, 55-K, **7-K**, **7-Kn**	
7-K:2k, **7-Kn:2k**	
7-P-K:2k	
7-pP_K:2k, 7-GFP_Kn	
7-P-A-K:2k	
7-pP-A_K:2k	

A plasmid encoding enhanced green fluorescent protein (GFP; KanRn as selection marker) was created using the non-restriction enzyme based NEBuilder Hifi DNA Assembly Cloning Kit (NEB, USA). *gfp* sequence was obtained from the plasmid pK7FWG2 (Karimi *et al.*, 2002) and it was under control of the *cpcB* promoter. This is the promoter of the gene encoding the c-phycocyanin beta subunit of *Synechocystis* sp. PCC 6803. A longer version of this promoter was reported to drive very high gene expression in *Synechocystis* sp. PCC 6803 and also a truncated version in known for high activity (Zhou *et al.*, 2014). The 3'UTR of the *gfp* ORF originates from the 3'UTR of the *psbA* ORF (Photosystem II protein D1) from HE10JO. The design principle for the plasmids for homologous recombination in *Phormidium lacuna* is illustrated in Table 4 in respect to the length of the homologous sequences and the promoter position. Conjugative plasmid pVZ321 with a RSF1010 based replicon (Zinchenko *et al.*, 1999) was kindly provided by the lab of A. Wilde (University of Freiburg, Germany).

Larger amounts of plasmid DNA for transformation into *Phormidium lacuna* were purified with NucleoBond Xtra Midi EF kit from Machery-Nagel (Germany). Manufacturer's protocol was changed to higher cultures volumes (300 ml) and glucose enriched growth medium was used (RB medium: 10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl, 2 g/l glucose, pH 7).

2.2 Cyanobacteria

The general procedure of cyanobacteria cultivation in this work is described here. Transformation related steps are listed in 2.3. *Phormidium lacuna* strains HE10JO, HE10DO, and GI09CO were used in this study as well as *Synechocystis* sp. PCC 6803. Culture of *Synechocystis* sp. PCC 6803 was obtained from S. V. Shestakov (Moscow State University, Russia). *Phormidium lacuna* strains were isolated and characterized by our workgroup (Nies *et al.*, 2017). All centrifugation steps with cyanobacteria were performed in refrigerated centrifuge (Z 383 K, Hermle, Germany, rotor: 220.80 V02 for 50 ml falcon tubes, rotor: 220.59 V06 for 1.5 or 2 ml tubes).

2.2.1 Cultivation

Cyanobacteria strains were cultivated under continuous illumination of 30 µmol m^{-2} s^{-1} white light from fluorescent tubes (Lumilux-DeLuxe L 18/954, Osram, Germany) at 23 °C. *Phormidium lacuna* was cultivated in the sea water media f/2 or f/2^{+}, *Synechocystis* sp. PCC 6803 was cultivated in the fresh water medium BG11/TES (Table 5). Standard cultivation volumes were 10 ml in 50 ml filter cap suspension culture flasks or 50 ml in 250 ml culture flasks (Greiner Bio-One, Austria). Suspension cultures were cultivated under continuous shaking at 70 rpm (TR-225, Infors, Switzerland). Transformants of *Phormidium lacuna* and *Synechocystis* sp. PCC 6803 were cultivated on various concentration of kanamycin sulfate (kanamycin hereafter), minimum 100 µg/ml kanamycin. In suspension culture supplemented with kanamycin, *Phormidium lacuna* was cultivated in f/2^{+} medium (f/2 medium with 10 fold increased concentration of nitrate and phosphate). In f/2^{+} medium,

Phormidium lacuna cultures are viable for several weeks or months. Medium was supplemented with 1.5 % Bacto Agar (BD Diagnostics, USA) for cultivation on agar plates.

Table 5: Media used for cyanobacteria cultivation with final concentrations. f/2 medium (Guillard & Ryther, 1962, Guillard, 1975): f/2 medium consist of artificial seawater and is supplemented with nitrate, phosphate, silicate, trace metal, and vitamin solution. Silicate was removed from f/2 composition since autumn 2016. $f/2^+$ medium is f/2 medium with tenfold increased nitrate and phosphate concentration. BG11/TES is BG11 (Stanier *et al.*, 1971) supplemented with TES buffer (100 mM, pH 8). BG11 stock (50x), thiamine hydrochloride, biotin, and cyanocobalamin (purity > 98%) were ordered from Sigma-Aldrich (USA). $MnCl_2$ (purity > 96%), $NaNO_3$, NaH_2PO_4, $FeCl_3$, $ZnSo_4$, $CuSO_4$, and Na_2MoO_4 were ordered from Merck (Germany).

f/2 medium, changes for $f/2^+$ in brackets		BG11/TES	
NaCl	0.41 M	$NaNO_3$	18 mM
$MgCl_2$	53 mM	$MgSo_4$	0.3 mM
Na_2SO_4	28 mM	$CaCl_2$	0.25 mM
$CaCl_2$	10 mM	K_2HPO_4	0.23 mM
KCl	9 mM	Na_2CO_3	0.19 mM
$NaHCO_3$	2.4 mM	H_3BO_3	48 µM
KBr	0.84 mM	citric acid	31 µM
H_3BO_3	0.49 mM	ferric ammonium citrate	23 µM
$SrCl_2$	90 µM	$MnCl_2$	9.2 µM
NaF	72 µM	EDTA disodium magnesium	2.8 µM
$NaNO_3$	0.88 mM (8.8 mM)	Na_2MoO_4	1.6 µM
Na_2SiO_3	0.11 mM	$ZnSO_4$	0.77 µM
NaH_2PO_4	36 µM (0.36 mM)	$CuSO_4$	0.32 µM
$FeCl_3$	12 µM	$Co(NO_3)_2$	0.17 µM
EDTA disodium	12 µM	TES	10 mM
$MnCl_2$	0.91 µM		
$ZnSo_4$	77 nM		
$CoCl_2$	42 nM		
$CuSO_4$	39 nM		
Na_2MoO_4	26 nM		
thiamine hydrochloride	0.3 µM		
biotin	2.1 nM		
cyanocobalamin	0.37 nM		

2.2.2 Determination of cell density

Cell density was measured photometrically via optical density at 750 nm (OD_{750}; Uvikon XS, NorthStar Scientific, UK). Prior to OD_{750} measurement *Phormidium lacuna* cultures were

homogenized due to filamentous growth and cell aggregates. OD_{750} measurements were performed to determine cell density and were applied for precise inoculation of *Phormidium lacuna* cultures. Cultures were homogenized mechanically by Ultraturrax (Silent Crusher M, Heidolph, Germany) with the dispersion tool 18F at 10,000 rpm for 3 min if not stated otherwise. Homogenization was performed alternatively with an ultrasound wand (UP100H Ultrasonic Processor, Hielscher, Germany) for 30 s at 30 kHz, 0.5 s burst, 0.5 s pause.

2.3 Transformation of *Phormidium lacuna*

The description of the transformation procedure of *Phormidium lacuna* is split into two parts. First, the general transformation procedure is described (2.3.1), and then the concrete protocols used during different phases of the optimization process are listed (2.3.2).

2.3.1 General transformation procedure

The basic principle of electroporation (EP) protocol procedure is the following: Cell aggregates of a *Phormidium lacuna* culture are loosened and filaments are shortened by homogenization. Cell density was measured by OD_{750}. Salt was removed from cell suspension by several washing steps. Subsequently, DNA and concentrated cell suspension were mixed and the electric pulse was applied. Cell suspension was directly transferred to suspension culture and cultivated two days. Afterwards, cells were harvested by centrifugation. Concentrated cell suspension was transferred to agar plates for selection of transformants.

This basic principle was always kept during the complete protocol development, but the conditions during the different steps were varied during the optimization procedure. Also with the discovery of natural transformation (NT), the basic principle stayed the same except that the electric pulse was removed from the protocol. In the following, the single steps of transformation protocol are described in detail for the common conditions. The different phases during protocol development are listed with the optimized parameters under 2.3.2. The conditions of single transformation experiments can be looked up in the electronic supplement (see transformation experiments; negative controls without plasmid DNA are not included in this list).

2.3.1 a) Homogenization and determination of cell density

Phormidium lacuna cultures (usually 2x 50 ml) were cultivated until reaching the desired cell density and were split in four 50 ml falcon tubes. Cell density was measured photometrically (OD_{750}). Prior to OD_{750} measurement *Phormidium lacuna* cultures were homogenized by Ultraturrax (see above). OD_{750} was measured directly after homogenization. *Phormidium lacuna* tends to build aggregates very fast after homogenization, but these aggregates can be resolved easily by shaking reaction tubes.

2.3.1 b) Centrifugation and washing

Homogenized cells were centrifuged at 6000 g or 7000 g at 4 °C for 15 min. Supernatant was discarded and cells were resuspended in washing solutions. Washing solutions were removed by subsequent centrifugation steps. Cells were washed two or three times. First washing solution was changed during protocol development (see below). Second and third washing steps (if applied) were performed with H_2O. Cooling during the washing procedure was intensified during protocol development. After washing cells were concentrated in smaller volumes as appropriate for respective experimental requirements. In 50 ml falcon tubes pellets of *Phormidium lacuna* can be too loose to remove supernatant sufficiently. For especially high cell density samples were therefore centrifuged again in 1.5 ml reaction to reach sufficient cell concentrations.

2.3.1 c) Electroporation

Plasmid DNA was added to concentrated cell suspensions. Plasmid stock concentrations were 1 – 2 µg/µl. Amounts of DNA used for every transformation experiment are documented (electronic supplement: transformation experiments). Different incubation times of plasmid in concentrated cell suspension were performed during protocol development. Cells were transferred into ice cold electroporation cuvettes (1 mm gap, VWR, USA) and the electric pulse was applied with the time constant protocol (Gene Pulser Xcell, Bio-Rad, USA). Adjustable settings are voltage and pulse duration. Resulting field strength is also influenced by the width of cuvette (in most cases 1 mm). Field strength and pulse duration leading to transformants are included in the transformation protocols (see 2.3.2). After pulse, cell samples were immediately transferred into 10 ml f/2 suspension culture and cultivated under standard conditions.

2.3.1 d) Identification of transformants

Cells were transferred on f/2 agar plates after 2 d regeneration: Samples were centrifuged (6000 g or 7000 g, 4 °C, 15 min) and resuspended in 1 ml supernatant. Cells were transferred on plates with 0, 70, and 120 µg/ml kanamycin, respectively, by drying a single drop of cell suspension in the middle of the agar plate. *Phormidium lacuna* transformants were selected by growth on selective media after two to four weeks. Detection of transformants can be alternatively be performed by application of light microscopy (DM750, Leica, Germany, camera: EC3), what enables faster identification of transformed cells. Transformed and untransformed cells were discriminated by color and filament shape on selective agar plates.

2.3.1. e) Validation of transformation with resistance and segregation of *kanR*

Transformants identified on agar plates were transferred into selective suspension culture ($f/2^+$, 100 µg/ml kanamycin). Initial cultivation in selective suspension culture is a first validation of

transformation. Transformants were also validated with PCR (see below) and segregation of selection marker in the genome was achieved with subcultivation on higher kanamycin concentrations.

2.3.1 f) Validation of transformation with PCR and segregation of *kanR*

Integration of homologous sequences into the genome of *Phormidium lacuna* was tested with PCR. The used primers are listed in above. Integration was monitored by different sizes of PCR fragments for *Phormidium lacuna* wild type and transformants due to integration of heterologous sequences or by specific primer binding sites on the integrated sequences. 1 µl of homogenized *Phormidium lacuna* samples (see 2.1.1) was used in 50 µl reactions with Taq or Q5 polymerase (NEB, USA). Reaction mixture was set up according to manufacturer's instructions (see also Table 2). Initial denaturation step of manufacturer's PCR program was set to 5 min. The duration of all other steps was adjusted if PCR results were not optimal and longer time for all steps increased efficiency of PCR in most cases.

2.3.2 Transformation protocols

In the following, the protocols used during optimization of the transformation procedure are listed. Differences between a protocol to the previous one are indicated with bold characters in the text. The different protocol cover the most common conditions tested during transformation optimization. The specific conditions of each experiment are listed in the electronic supplement (see transformation experiments).

2.3.2 a) Electroporation protocol I

Culture of *Phormidium lacuna* (2x 50 ml) was incubated until desired cell density was reached. This was determined by optical impression. Cell suspension was transferred into falcon tubes and kept on ice until electroporation. Cell suspension was homogenized by Ultraturrax (see 2.3.1). Subsequently, OD_{750} was measured. Cell cultures with an OD_{750} of 0.15 – 0.4 were used. OD_{750} was not documented regularly. Cell suspension was centrifuged (7000 g, 4 °C, 15 min) and supernatant was discarded. Cells were resuspended in 20 ml washing buffer (Tricine 2 mM, EDTA 2 mM, pH 8) per falcon. Cell suspension was centrifuged again (7000 g, 4 °C, 15 min) and supernatant was discarded. Cells were resuspended in 20 ml H_2O per falcon and centrifuged again (7000 g, 4 °C, 15 min). This washing step with H_2O was repeated once. Afterwards, most supernatant was removed and cells were resuspended in the residual volume. Concentrated cell suspension was transferred into 1.5 ml tubes. If necessary, concentrated cell suspension was centrifuged again (7000 g, 4 °C, 15 min) to reach the required concentration. 90 – 100 µl concentrated cell suspension was used for one transformation approach. Concentrated cell suspension was mixed with plasmid DNA (10 – 20 µg). The equivalent volume of water was mixed with concentrated cell suspension as negative control. Transformation approaches were electroporated in the same order as cell-DNA mixtures were prepared. Additional incubation time prior to electroporation was dependent on the experiment. The adjustable electroporation

parameter of the applied time constant protocol were 2.5 – 5 kV cm^{-1}, 3 – 4 ms. Cells were transferred into suspension culture flask with 10 ml f/2 medium immediately after the electric pulse and cultivated for 2 d under standard conditions. Subsequently, cell suspension was transferred into 50 ml falcon tubes and centrifuged (7000 g, 4 °C, 15 min). Cells were resuspended in 1 ml supernatant and transferred on agar plates as described above. Transformants were selected and validated as described above (see 2.3.1) without use of microscopy.

2.3.2 b) Electroporation protocol II

Culture of *Phormidium lacuna* (2x 50 ml) was incubated until desired cell density was reached. This was determined by optical impression. Cell suspension was transferred into falcon tubes and **kept on ice water** until electroporation. Cell suspension was homogenized by Ultraturrax (see 2.3.1). Subsequently, OD_{750} was measured. Cell cultures with an **OD_{750} of 0.08 – 0.3** were used. **OD_{750} was documented regularly.** Cell suspension was centrifuged (**6000 g**, 4 °C, 15 min) and supernatant was discarded. Cells were resuspended in 20 ml **H_2O or EDTA solutions in differing concentration (pH 8, max 0.9 mM) per falcon**, depending on the experimental settings. Cell suspension was centrifuged again (**6000 g**, 4 °C, 15 min) and supernatant was discarded. Cells were resuspended in 20 ml H_2O per falcon and centrifuged again (**6000 g**, 4 °C, 15 min). Afterwards, most supernatant was removed and cells were resuspended in the residual volume. Concentrated cell suspension was transferred into 1.5 ml tubes. Concentrated cell suspension was centrifuged again (**6000 g**, 4 °C, 15 min) **regularly** to reach the required concentration. 100 µl concentrated cell suspension was used for one transformation approach. Concentrated cell suspension was mixed with plasmid DNA (**1 – 30 µg**). The equivalent volume of water was mixed with concentrated cell suspension as negative control. Transformation approaches were electroporated in the same order as cell-DNA mixtures were prepared. Additional incubation time prior to electroporation was dependent on the experiment. The adjustable electroporation parameter of the applied time constant protocol were 2.5 – 5 kV cm^{-1}, 3 – 4 ms. Cells were transferred into suspension culture flask with 10 ml f/2 medium immediately after the electric pulse by **flushing the electroporation cuvette with media**. Cells were cultivated for 2 d under standard conditions. Subsequently, cell suspension was transferred into 50 ml falcon tubes and centrifuged (**6000 g**, 4 °C, 15 min). Cells were resuspended in 1 ml supernatant and transferred on agar plates as described above. Transformants were selected and validated as described above (see 2.3.1).

2.3.2 c) Electroporation protocol III

Culture of *Phormidium lacuna* (2x 50 ml) was incubated until desired cell density was reached. **Cultures were inoculated to reach a cell density of an OD_{750} between 0.25 and 0.35**. Cell suspension was transferred into falcon tubes and kept on ice water until electroporation. Cell suspension was homogenized by Ultraturrax (see 2.3.1). Subsequently, OD_{750} was measured. Cell cultures with an **OD_{750} of 0.25 – 0.35** were used. OD_{750} was documented regularly. Cell suspension

was centrifuged (6000 g, 4 °C, 15 min) and supernatant was discarded. Cells were resuspended in 20 ml H_2O per falcon. Cell suspension was centrifuged again (6000 g, 4 °C, 15 min) and supernatant was discarded. Cells were resuspended in 20 ml H_2O per falcon and centrifuged again (6000 g, 4 °C, 15 min). Afterwards, most supernatant was removed and cells were resuspended in the residual volume. Concentrated cell suspension was transferred into 1.5 ml tubes. Concentrated cell suspension was centrifuged again (6000 g, 4 °C, 15 min) regularly to reach the required concentration. 100 µl concentrated cell suspension was used for one transformation approach. Concentrated cell suspension was mixed with plasmid DNA (1 – 30 µg). The equivalent volume of water was mixed with concentrated cell suspension as negative control. **Mixed samples were directly used for electroporation.** The adjustable electroporation parameter of the applied time constant protocol were **3 kV cm^{-1}, 4 ms**. Cells were transferred into suspension culture flask with 10 ml f/2 medium immediately after the electric pulse by flushing the electroporation cuvette with media. Cells were cultivated for 2 d under standard conditions. Subsequently, cell suspension was transferred into 50 ml falcon tubes and centrifuged (6000 g, 4 °C, 15 min). Cells were resuspended in 1 ml supernatant and transferred on agar plates as described above. Transformants were selected and validated as described above (see 2.3.1).

2.3.2 d) Natural transformation protocol

Culture of *Phormidium lacuna* (2x 50 ml) was incubated until desired cell density was reached. Cultures were inoculated to reach a cell density of an OD_{750} between 0.25 and 0.35. Cell suspension was transferred into falcon tubes and kept on ice water until the addition of DNA. Cell suspension was homogenized by Ultraturrax (see 2.3.1). Subsequently, OD_{750} was measured. Cell cultures with an OD_{750} of 0.25 – 0.35 were used. OD_{750} was documented regularly. Cell suspension was centrifuged (6000 g, 4 °C, 15 min) and supernatant was discarded. Cells were resuspended in 20 ml H_2O per falcon. Cell suspension was centrifuged again (6000 g, 4 °C, 15 min) and supernatant was discarded. Cells were resuspended in 20 ml H_2O per falcon and centrifuged again (6000 g, 4 °C, 15 min). Afterwards, most supernatant was removed and cells were resuspended in the residual volume. Concentrated cell suspension was transferred into 1.5 ml tubes. Concentrated cell suspension was centrifuged again (6000 g, 4 °C, 15 min) regularly to reach the required concentration. 100 µl concentrated cell suspension was used for one transformation approach. Concentrated cell suspension was mixed with plasmid DNA (1 – 30 µg). The equivalent volume of water was mixed with concentrated cell suspension as negative control. **Cells were directly transferred into suspension culture** flask with 10 ml f/2 medium immediately. Cells were cultivated for 2 d under standard conditions. Subsequently, cell suspension was transferred into 50 ml falcon tubes and centrifuged (6000 g, 4 °C, 15 min). Cells were resuspended in 1 ml supernatant and transferred on agar plates as described above. Transformants were selected and validated as described above (see 2.3.1).

2.4 Ethanol detection

Phormidium lacuna HE10DO transformants for heterologous ethanol production encoding for one or both enzymes (PDC or PDC/ADH) were tested for ethanol in cultures supernatant.

2.4.1 Enzyme assay

The detection of ethanol in an aqueous solution is measured indirectly by the back reaction towards acetaldehyde catalyzed by the ADH enzyme of *Saccharomyces cerevisiae* (Sigma-Aldrich, USA). The coupled increase of NADH (see also Figure 2) is monitored photometrically with the increase of OD_{340} (V-550, Jasco, Germany).

The supernatant of a cyanobacteria culture was centrifuged at 6000 g, 4 °C, 15 min and 1 ml of supernatant was transferred to a new reaction tube. The reaction solution was mixed together in a photometer cuvette in the volumes listed in Table 6. All solutions except the ethanol containing ones were mixed together and the OD_{340} development was monitored continuously for ca. 2 min. OD_{340} can change slightly in the beginning. Subsequently, ethanol containing solutions (standard solution or samples supernatant) were added and OD_{340} development was monitored until 10 min total run time was completed.

Table 6: Reaction solution for the enzymatic assay of ethanol detection. Sodium pyrophosphate, phosphoric acid, and NAD (purity > 96.5%) was ordered from Sigma-Aldrich (USA). Ethanol was ordered from VWR (USA). BSA: bovine serum albumin.

Volume [ml]	Solution	Concentration/preparation	required solutions
1.3	Sodium phosphate buffer (pH 8.8)	50 mM	50 mM sodium pyrophosphate, pH 8.8 adjusted by 9.5% (v/v) phosphoric acid
1	NAD solution	15 mM	
0.1	ADH working solution	50 µl ADH stock solution 25 ml Enzyme diluent	ADH stock solution: 1 mg/ml ADH in 10 mM sodium phosphate buffer (pH 7.5)
			Enzyme diluent: 10 mM sodium phosphate buffer (pH 7.5), 1 mg/ml BSA
2.4 (total)	First OD_{340} measurement [2 min]		
0.6	ethanol dilution	various	
or			
0.25	culture supernatant		
0.35	H_2O		
3 (total)	Second OD_{340} measurement [8 min]		

2.4.2 Gas chromatography

Synechocystis sp. PCC 6803 cultures were cultivated in BG11/TES media, *Phormidium lacuna* HE10DO cultures in $f/2^+$, $f/2^+$ with Glucose (1 mM), and f/2 media. The promoter *petJ* of the *Synechocystis* sp. PCC 6803 transformant petJ PA is induced by copper depletion: 10 µl bathocuproine (100 µM) were added to *Synechocystis* sp. PCC 6803 cultures. Transformants were cultivated with medium containing 100 µg/ml kanamycin. All samples were inoculated with the same OD_{750} (0.01) and cultivated 7 d. HE10DO cultures were homogenized prior to inoculation. The conditions for every sample are listed in Table 7. All samples in Table 7 were prepared three times independently for the determination of ethanol production by GC

Table 7: Samples for gas chromatography measurements. Gluc: additional Glucose (1 mM). Incubation in darkness: Samples were wrapped in Aluminum foil on day 4.

Organism	Genotype	Medium	Kanamycin [µg/ml]	Incubation in darkness
Synechocystis sp. PCC 6803	WT	BG11/TES	0	
	petJ PA	BG11/TES	100	
Phormidium lacuna HE10DO	WT	$f/2^+$	0	
	WT	$f/2^+$, Gluc	0	
	WT	f/2	0	
	WT	$f/2^+$	0	yes
	7-Kn:2k	$f/2^+$	100	
	7-Kn:2k	$f/2^+$, Gluc	100	
	7-Kn:2k	f/2	100	
	7-Kn:2k	$f/2^+$	100	yes
	7-P-A-K:2k	$f/2^+$	100	
	7-P-A-K:2k	$f/2^+$, Gluc	100	
	7-P-A-K:2k	f/2	100	
	7-P-A-K:2k	$f/2^+$	100	yes
	7-P-K:2k	$f/2^+$	100	
	7-pP_K:2k	$f/2^+$	100	

After cultivation, 2 ml supernatant was transferred into reaction tubes and centrifuged (6000 g, 4 °C, 15 min). Half of the volume was transferred into a new reaction tube. Centrifugation step was repeated once. The remaining bacterial culture was homogenized with ultrasound and the OD_{750} was measured. The organic composition of centrifuged samples was measured by gas chromatography (GC; HP 6890 GC system, Agilent, USA) equipped with a flame ionization detector (FID), and split/splitless inlet. A DB-wax column (Agilent, USA) with dimensions 30 m (length) x 0.32 mm (internal diameter) x 0.25 µm (film thickness) was used (column was shortened to 29.7 m before experiments). The injection of

1 µl sample volume was performed manually. Split ratio was 1:10 for the relevant measurements. Front inlet temperature was 130 °C and helium was used a carrier gas. An average velocity of 35 cm/s was used with constant flow and constantly adjusted pressure. The FID's temperature was set to 250 °C. The oven was heated to an initial temperature of 45 °C. This temperature was hold for 2 min, then temperature was increased with 50 °C/min until 220 °C and held for 5 min. GC primary signal was processed by the software ChromStar (version 7.0.12; SCPA GmbH, Germany). Ethanol signal was determined with peak retention time (2.74 – 2.76 s) and peak area of samples was set in relation to peak area of ethanol standard solutions (Figure 22).

2.5 BLASTp search for natural transformation factors in cyanobacteria

Homologous sequences for proteins of natural transformation factors (NTFs) were predicted by bioinformatics tools. NTFs are proteins that were proven experimentally to be essential in *Synechocystis* sp. PCC 6803 for NT or that are considered to play a central role in NT due to their annotation.

A list of the NTFs used in this study is found in Table 13 in 3.4.2. Protein sequences of the predicted ORFs were obtained from the NCBI database and saved one TXT file.

A selection of cyanobacteria with sequenced genomes Table 12 in 3.4.1 was made using the NCBI database[1]. The criteria for the selection were to cover the majority of the cyanobacterial phylogenetic tree and to have a broader representation of cyanobacteria capable of NT and their closer relatives. All relevant proteins of an organism were downloaded in one file in FASTA format. The sequence information was obtained from the NCBI database and can be identified by their taxonomy ID and in most cases also by their RefSeq number (supplement: Table 18).

The offline commando line based NCBI tool BLAST+ (version: 2.7.1, basic local alignment search tool) was used to align the collection of NTFs with every cyanobacteria genome of the collection (Camacho *et al.*, 2009). The used command lines are listed below. The part in brackets of a command line indicates the specific file name.

The unpacked protein collection files were converted into databases with the command line:

makeblastdb.exe -in [protein-collection.fasta] *-input_type fasta -dbtype prot -out* [protein-database]

The NTF collection in the TXT file was aligned with the protein databases using BLASTp. TXT output was used for initial analysis; XML output was used for processing the data in excel. Following commands were used.

For TXT output:

blastp.exe -num_alignments 1 -query [NTF.txt] *-db* [protein-database] *-out* [alignment.txt]

[1] https://www.ncbi.nlm.nih.gov/genomes/GenomesGroup.cgi?taxid=1117 (22.08.2018)

For XML output:

blastp.exe -query [NTF.txt] *-db* [protein-database] *-max_hsps 1 -max_target_seqs 1 -out* [alignment.xls] *-outfmt 5*

Detailed instructions for BLAST+ can be found here on the NCBI homepage[2]. Besides the NTFs of *Synechocystis* sp. PCC 6803 also the NTFs of other naturally competent cyanobacteria (NCC) with a sequenced genome were used for BLASTp analysis. Therefore the best hits of the BLASTp with the NTF list of *Synechocystis* sp. PCC 6803 were used to generate the new NTF list for the respective NCC (supplement: Table 18).

BLAST results were analyzed using excel commands for smallest value, biggest value, mean value and its standard deviation. Furthermore, different outcomes (bit score, E value, alignment length) were set in relation to each other or were summarized for analysis. Exact condition for comparison is mentioned in the relevant analysis in the results section.

[2] https://www.ncbi.nlm.nih.gov/books/NBK279690/ (22.08.2018)

3 Results

Central objective of this work is to make *Phormidium lacuna* accessible for recombinant application and to characterize its potential in biotechnology by the heterologous production of ethanol. The establishment of a transformation method for *Phormidium lacuna* is an essential prerequisite for this objective. During the process of the development of an electroporation method it was discovered that *Phormidium lacuna* is naturally transformable. Natural competence is only described for a small selection of cyanobacteria and this work is the first report in the order Oscillatoriales. The finding of natural transformation is set in relation to other cyanobacteria by bioinformatics. The results are ordered and divided into the following sections:

1) This part focuses on the development of the transformation method of *Phormidium lacuna*. It starts with the initial electroporation protocol, proceeds with the overcoming of intermediate viability problems and the subsequent optimization of the electroporation protocol. Furthermore, the discovery of natural competence of *Phormidium lacuna* is documented as well as the switch to a natural transformation protocol.

2) Findings that arose during protocol development are described here. Transformants of *Phormidium lacuna* show considerable high resistance towards the selection marker kanamycin. In addition, based on PCR results it is shown that *Phormidium lacuna* is polyploid. The high antibiotic resistance allows fast segregation of the selection marker into the genome of *Phormidium lacuna*. Retardation in cell propagation during antibiotic selection revealed that *Phormidium lacuna* strain HE10JO is affected by an environmental contamination.

3) The genes for ethanol production are expressed in transformants for characterizing the biotechnology potential of *Phormidium lacuna*. Ethanol production of these transformants is monitored. Possible reasons for the non-detectable difference in the ethanol production between *Phormidium lacuna* wild type and different *Phormidium lacuna* transformants are reflected here and in the discussion section.

4) The surprising finding of natural competence in a cyanobacterium within the order Oscillatoriales is set into relation to the whole phylum of cyanobacteria. A homology based comparison of natural competence factors between different cyanobacteria is used to make a prediction of promising candidates to test for natural transformation.

3.1 Transformation of *Phormidium lacuna*

The main part of this work deals with the establishment of the transformation of *Phormidium lacuna*. This is the initial and an essential step to make this organism genetically accessible for biotechnological application. Any further work, which engages heterologous gene expression or the generation of mutants, relies on this initial step. Since *Phormidium lacuna* is a hardly characterized organism in many respects, the establishment of new protocols presents a challenge because also unknown organism specific properties and requirements have to be considered.

3.1.1 Initial protocol

Electroporation was the method of choice for the transformation of *Phormidium lacuna*, since electroporation is a universal method for gene transfer. Also other cyanobacteria of the order Oscillatoriales can be transformed by this method (see introduction). Electroporation (EP) protocols of three cyanobacteria were considered for the establishment of a transformation protocol for *Phormidium lacuna*: *Synechococcus elongatus* (Muhlenhoff & Chauvat, 1996) as a species that is well established in cyanobacteria biotechnology and *Oscillatoria* MKU 277 (Ravindran *et al.*, 2006) and *Arthrospira platensis* (Toyomizu *et al.*, 2001) as two representatives of the same order as *Phormidium lacuna*. From these three protocols, preliminary experiments, and regular lab experience in the handling with *Phormidium lacuna*, the initial protocol (EP protocol I, see 2.3.2) was developed.

The common principles of all EP protocols in this study are the following: Cell suspension was cultivated to a certain cell density, which was quantified via optical density at 750 nm (OD_{750}) after cell suspension was homogenized. Prior to any treatment *Phormidium lacuna* cell suspension had to be homogenized due to its filamentous growth and its strong tendency to form aggregates. Homogenous cell suspension was required for OD_{750} measurements and for even cell distribution. Salt was removed from the cell suspension with subsequent washing steps. Afterwards, cells were concentrated and mixed with plasmid DNA. Different incubation times of the mixture of DNA and cells were tested during protocol development. Then, cells were electroporated and subsequently transferred into suspension culture. After 2 d incubation for regeneration and expression of the selection marker, cells were transferred on agar plates. Transformants were identified by growth on selective plates. Transformation was validated by growth in selective suspension culture at comparable and higher kanamycin concentration. Furthermore, homologous recombination events required for transformation were monitored by PCR.

Phormidium lacuna grows in filaments and is capable of gliding motility. Thus, transformants do not form colonies but spread over the complete agar plate with time. It is hard to detect transformed filaments by spreading of the cell suspension on the whole agar plate. Therefore, *Phormidium lacuna* culture was transferred on agar plates by drying a single drop of cell suspension in the middle of the agar plate. *Phormidium lacuna* transformants could be selected by propagation on selective media after two to four weeks. The selection time was rather long, because *Phormidium lacuna* wild type

(WT) can tolerate 70 µg/ml kanamycin on agar plates for some time. Accelerated selection of transformants was conducted on agar plates with concentration of 120 µg/ml kanamycin. But it was also observed that the higher kanamycin concentration can inhibit propagation of transformants during prolonged cultivation periods on agar plates (see also Figure 10 in 3.1.2). This finding is discussed in respect with other results in the discussion section (see 4.1.2). Schematic growth of *Phormidium lacuna* on non-selective and selective plates is shown in Figure 5. Without selective pressure, filaments of *Phormidium lacuna* spread over the whole agar plate during cultivation. With selective pressure cells start to die after ca. one week. Depending on the kanamycin concentration and cell viability it can last up to four weeks or longer until all untransformed cells have died. If transformation was successful, filaments of transformants can be identified.

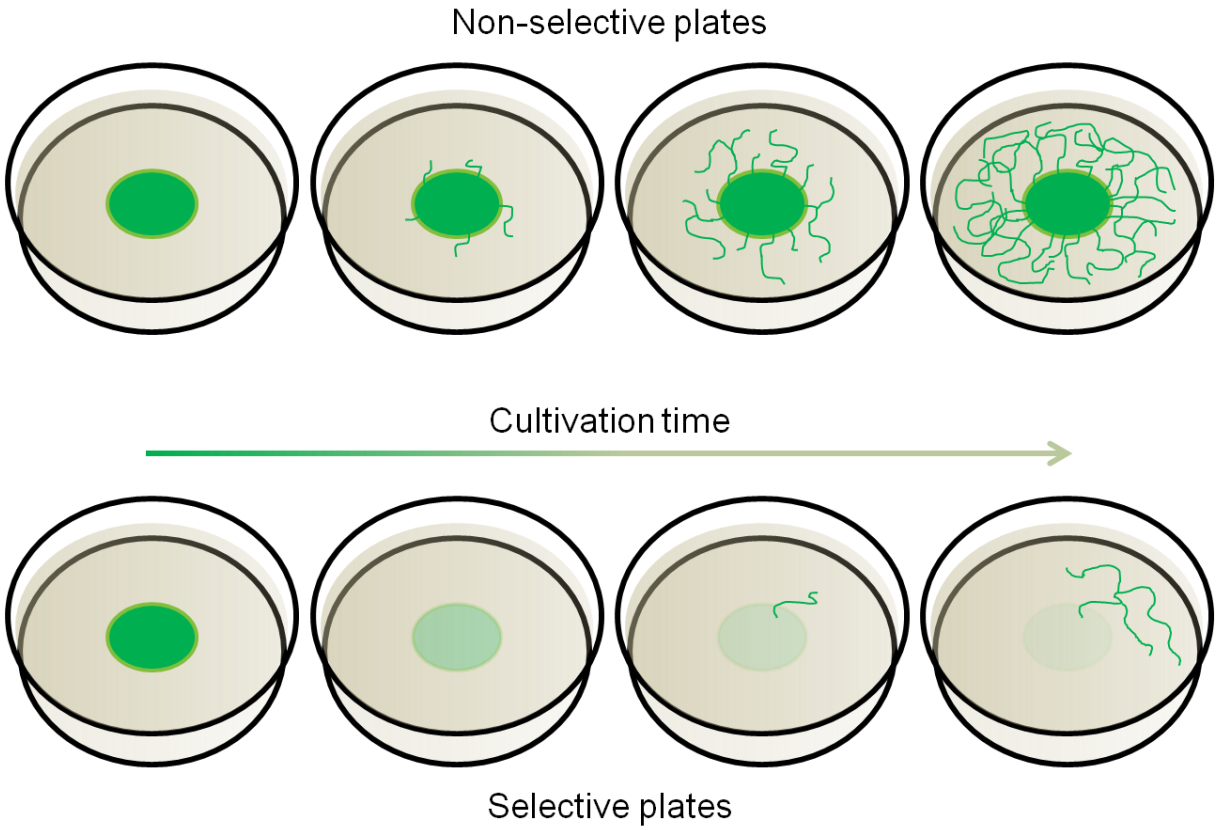


Figure 5: Scheme of cultivation of *Phormidium lacuna* on non-selective and selective f/2 agar plates over time. Transformants can be identified by the growth on selective plates.

When delivering a gene of interest to a bacterium, two localizations can be considered: Either integration into the host's genome or encoding on a plasmid (of native or heterologous origin). For the transformation of *Phormidium lacuna* homologous recombination into the chromosome was considered because of the following reasons: So far, there is no information about native plasmids in *Phormidium lacuna*. Although the use of self-replicating plasmids has been investigated in other cyanobacteria, it is not know whether they would replicate also in members of the genus *Phormidium*. Integration into the genome by homologous recombination, a general mechanism for DNA repair and

recombination, was thus considered more promising and also more stable than the transformation with a self-replicating plasmid.

For transformation of *Phormidium lacuna*, the kanamycin resistance operon (*kanR*) operon should be integrated into the HE10JO genome by homologous recombination. Therefore, three different loci were chosen that were not considered essential (see below). The selection marker was integrated in the open reading frames (ORFs) annotated (Nies *et al.*, 2017) to code for a phytochrome, a photoreceptor with a two component sensor histidine kinase activity (5'-3'; 02_124; phl#1409; WP_087705573), for HypE, a protein involved in Ni-Fe hydrogenase maturation (5'-3'; 55_26; phl#3443; WP_087711477) and a hypothetical protein (3'-5'; 07_37; phl#3878; WP_087706519)[3]. The hypothetical protein phl#3878 was selected because it was considered a hydrogenase. This was supported by BLASTp[4] analysis (default settings, non-redundant protein sequences database), which revealed homology with a hydrogenase of *Arthrospira platensis* (bit score: 244, E value: 3e-73, query cover: 92%; WP_035759913.1). The automatic annotation of the genome of *Phormidium lacuna* HE10JO (Nies *et al.*, 2017) was performed later and led to a differing annotation without a distinct function ("possible surface protein, responsible for cell interaction; contains cell adhesion domain and ChW-repeats"). Thus, the annotation of phl#3878 was not definite but the hydrogenase annotation was still favored.

A position inside the ORF of the proteins mentioned above was chosen as integration side for the selection marker. These positions in the genome are generally accessible for transcription and the selected ORFs were considered not essential. Mutants of the cyanobacterial phytochromes do not show a phenotype under low intensities of white light in *Synechocystis* sp. PCC 6803 (Fiedler *et al.*, 2004). Hydrogenases were not considered essential since the play a role in the regeneration of reduction equivalents under anaerobic conditions or in nitrogen fixation via the synthesis or metabolism of H_2 (Tamagnini *et al.*, 2007). Thus, under standard culture conditions (aerobic and oxygenic photosynthesis, no nitrogen starvation) hydrogenases should not play a critical role in metabolism. The resulting plasmids were abbreviated 2-K, 55-K, and 7-K, respectively. The number indicates the respective locus and "K" stands for the *kanR* operon.

In 2.3.2 the conditions leading to transformants in three independent experiments in the year 2016 are summarized in EP protocol I. Tested conditions, which did not result in transformants, were not included in the initial EP protocol. The settings of each evaluable transformation experiment performed in this study with *Phormidium lacuna* are documented in the electronic supplement (see transformation experiments). A subsection of the agar plates of the first successful transformation are shown in Figure 6. This picture is representative for the selection of transformants.

[3] Bracket information: 1) Resistance gene inserted in the same (5'-3') or opposite (3'-5') transcription direction as the ORF of the insertion side. 2) Name of the homologous sequences. 3) Name of the encoded protein in the integration site (Nies *et al.*, 2017) 4) GenBank accession number of the protein.

[4] https://blast.ncbi.nlm.nih.gov/Blast.cgi (08.10.2018)

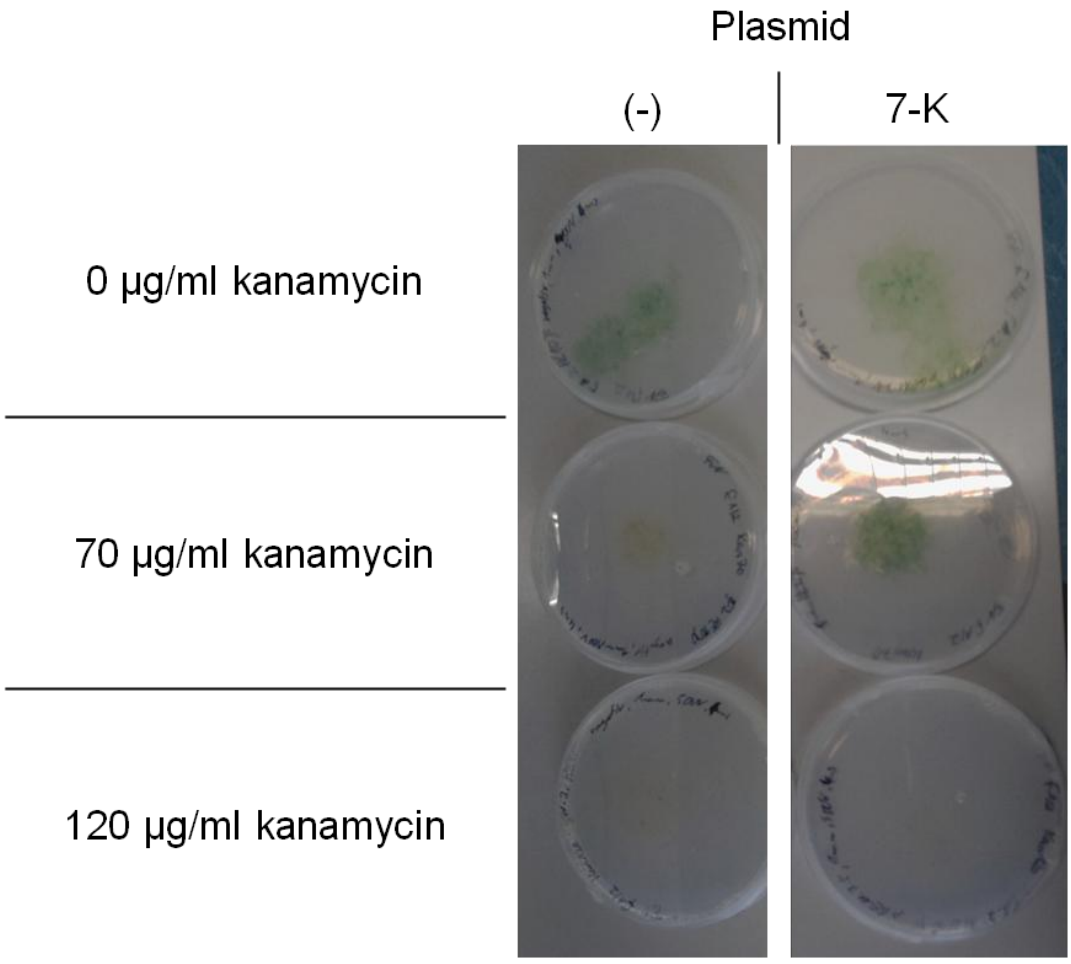


Figure 6: Picture of the f/2 agar plates of the first successful transformation of HE10JO. Transformants were selected for plasmid for locus 07_37, 7-K, on plates with 70 µg/ml kanamycin. No cell propagation of transformants could be observed on plates with 120 µg/ml kanamycin at this time point of the selection procedure. Negative control did not show cell propagation on f/2 agar plates with kanamycin.

In Figure 6 results from the transformation of *Phormidium lacuna* HE10JO with the plasmids 7-K and the negative control are shown. Transformants were selected only for the plasmid 7-K. Figure 6 shows an early state of the selection process, in which transformants were only selected for 70 µg/ml kanamycin. After longer incubation, transformants could also be selected on 120 µg/ml kanamycin in the respective experiment. Transformations with the plasmids 2-K and 55-K were also tested in the first transformation experiment but it did not result in transformants. This means that only locus 07_37 was tested successfully for homologous transformation in *Phormidium lacuna* HE10JO in the first transformation experiment. Due to the finding that only homologous recombination with plasmid 7-K resulted in transformants, this plasmid was used in the majority of the subsequent experiments. The other two loci were only tested once again in a later transformation experiment but without resulting in transformants. Thus, the locus 07_37 was the only locus, for which homologous recombination worked in this whole study.

Transformants for 7-K were only selected with the electroporation parameters 5 kV cm^{-1}/ 4 ms (1 mm EP cuvette) in the first transformation experiment, while no transformants were selected with the parameters 5 kV cm^{-1}/ 2 ms (1 mm and 2 mm EP cuvettes). Together with two subsequent transformation experiments, the conditions leading to transformation are summarized in the EP protocol I (see 2.3.2). Conditions not included in the initial EP protocol encompass field strength that were not in the range of 2.5 – 5 kV cm^{-1}, electroporation cuvettes with 2 mm gap, and one unsuccessful attempt of natural transformation. For conditions of single transformation experiments see electronic supplement. In Figure 7 the transformation efficiency with the plasmid 7-K is illustrated for all tested conditions and the conditions leading to transformants (EP protocol I). Although only conditions resulting in transformants are included in the initial protocol, reproducibility of the transformation protocol was rather low. For instance, approaches with the electroporation parameter 5 kV cm^{-1}/ 4 ms, which resulted in transformants in the first experiment, did not result in transformants in the other two experiments. The low reproducibility is correlated with rather low transformation efficiency (see below).

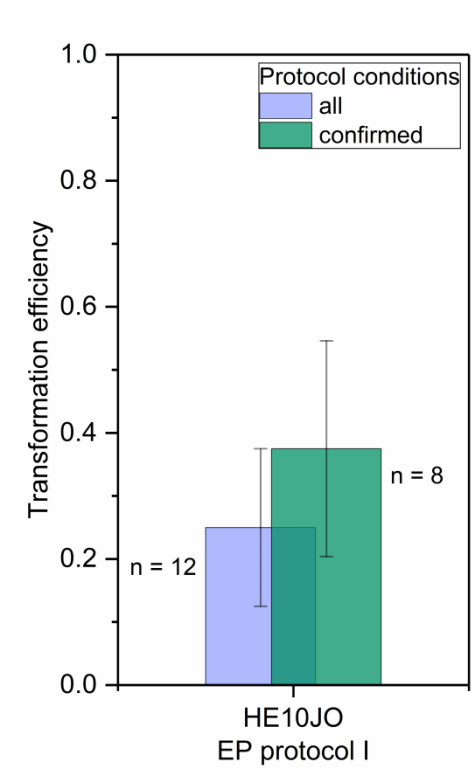


Figure 7: Transformation efficiency of the first three transformations of *Phormidium lacuna* HE10JO in 2016. Transformation efficiency was calculated by the number of approaches that resulted in transformants divided by the total number of approaches (n) in the respective experiments. In a positive transformation event descendants of one or more filaments were cultivable in selective suspension culture at low kanamycin concentrations (100 – 250 µg/ml) initially. Furthermore, the integration of the selection marker into the genome was verifiable with PCR and the transformants were cultivable on higher kanamycin concentration (> 500 µg/ml). Blue bar: All approaches tested with the 7-K plasmid. Green bar: All approaches with conditions that resulted once into transformants (confirmed conditions of electroporation protocol I). Number of monitored experiments is indicated. Error bars show standard error. Significance: T-test error probabilities are above threshold (5%).

As mentioned above *Phormidium lacuna* does not form colonies on agar plates due to filamentous growth and gliding motility (Figure 5). Without colonies transformation cannot be quantified by colony forming units per µg DNA. Therefore, the number of approaches in the respective experiments, which resulted in transformants, was divided by the total amount of approaches in the respective experiments. For instance, in three independent experiments in 2016 twelve transformation approaches with plasmid 7-K were performed and three of them resulted in transformants. Thus, transformation efficiency is 25% ± 13% (error is indicated by standard error). If only the conditions of the EP protocol I are considered, only 8 approaches in the three experiments are included into calculation and transformation efficiency is 37.5% ± 17% (Figure 7). In the following, it is distinguished between transformation rate (transformation events in one approach in relation to inserted amount of plasmid DNA), which could not be calculated for *Phormidium lacuna*, and transformation efficiency, which indicates the percentage of a number of approaches that resulted in transformants.

It was shown by Finn Zaiß (bachelor thesis) that the EP protocol I can be transferred to two other *Phormidium lacuna* strains, GI09CO and HE10DO. These strains have the same 16S rRNA gene sequence as strain HE10JO and only minor differences in the phytochrome gene sequence and thus are regarded as the same species (Nies *et al.*, 2017).

The propagation of filaments on selective agar plates was the first indication of a positive transformation event. There were two criteria for the validation of a transformation event: 1) Selected filaments were transferred into selective suspension culture with elevated phosphate and nitrate concentrations ($f/2^+$ medium, 100 – 250 µg/ml kanamycin). Transformants had to be cultivable at this concentration for at least 8 weeks and also be cultivable at higher kanamycin concentration (see also 3.2). 2) The integration of the kanamycin gene into the *Phormidium lacuna* genome was demonstrated by PCR. The transformation by 7-K was monitored by using the Primers F25 and F28 that have binding sites in the genome up- or downstream of the integration site, respectively (Figure 8). While for HE10JO WT only the shorter 1213 bp PCR product was obtained, for HE10JO transformants also the longer 2560 bp PCR product was detected. This PCR test and the cultivation of transformants in selective suspension culture were regarded as sufficient for validation of *Phormidium lacuna* transformation. Both approaches were used in all subsequent experiments to verify the transformation and integration of the *kanR* operon into the *Phormidium lacuna* genome.

Notably, for transformants of the first successful transformation, which were four weeks in selective suspension culture, only the recombinant PCR product was detectable (T1). In contrast, for transformants of the second experiment (T2) with five days cultivation under selective pressure also the shorter PCR product without *kanR* operon as insert was obtained (Figure 8). This was the first indication that *Phormidium lacuna* is polyploid. Further investigations on polyploidy and transformation of *Phormidium lacuna* are listed in 3.2.

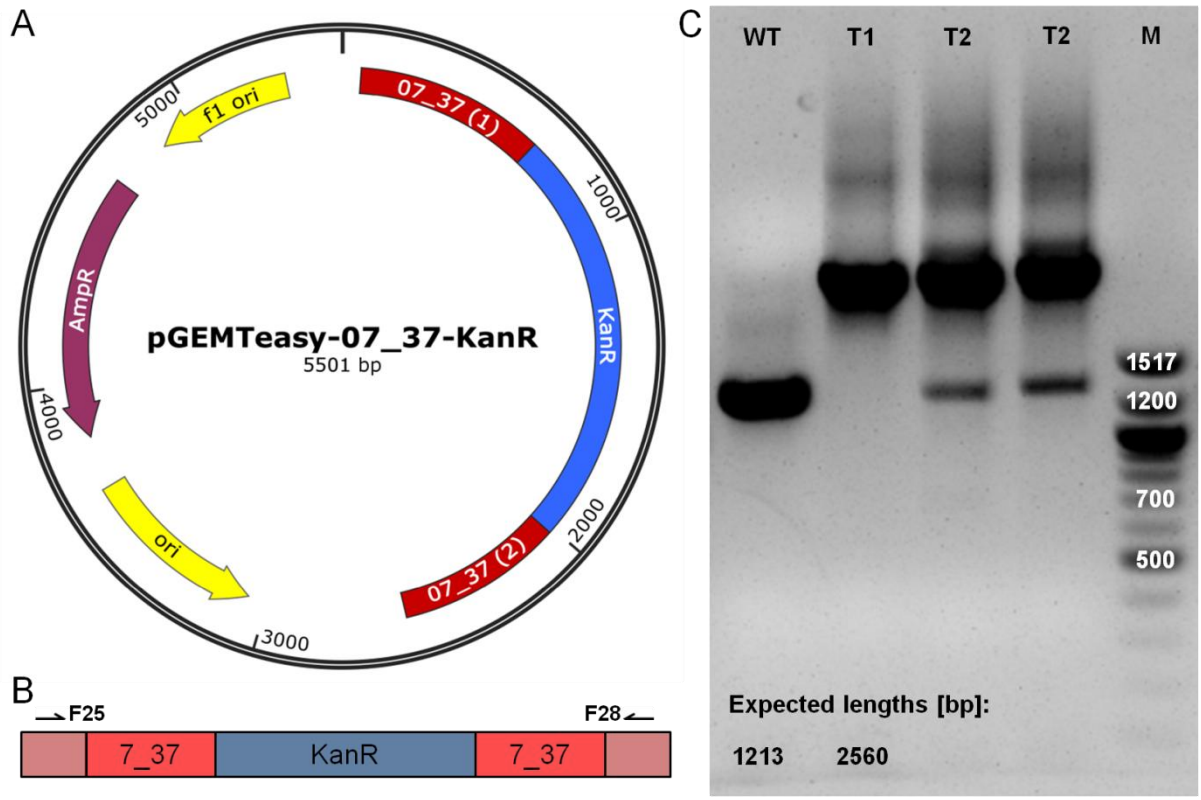


Figure 8: Validation of transformation. A) 7-K plasmid (full name: pGEMTeasy-07_37-KanR): red - homologous sequences of the 07_37 locus, blue - kanamycin resistance operon, yellow - origin of replication (f1 - bacteriophage origin, other - pUC origin for *Escherichia coli*), purple - ampicillin resistance operon. **B) Integration site of the 7-K plasmid with primer binding sites.** red - homologous sequences encoded on the vector, pale red - *Phormidium lacuna* chromosome, blue - kanamycin resistance gene. Primer pair: F25/F28 covering whole insertion site. **C) Validation of transformants by PCR.** Agarose gel for the PCR with the primer pair that covers the full insert for *Phormidium lacuna* WT and transformants of the first (T1) and second (T2) experiment. M: 100 bp DNA ladder (NEB, USA). Integration of the kanamycin resistance operon into the genome of Phormidium lacuna was indicated by the bigger PCR product (2560 bp).

An additional PCR approach was performed that proves the integration of the *kanR* operon into the genome by the primer binding in the *kanR* ORF (open reading frame). The primer pairs h1, h2, K, and A (primer pair A: F31/F32, binding site in AmpR, ampicillin resistance operon) were tested (Figure 20 in the supplement). For primer pair h1, h2, and K, but not for primer pair A, PCR products of the correct size could be detected. The primer pairs h1 and h2 have one binding site in the *KanR* operon, while the primer pair K has two binding site in the *kanR* operon. The results in Figure 8 and Figure 20 show that exclusively the *kanR* operon was integrated as proposed in the 07_37 locus by homologous recombination, based on the expected length of the PCR products. The origin of replication of the pGEMTeasy vector promotes high copy number in *Escherichia coli*. This origin of replication is considered not functional in cyanobacteria as no origin of replication is known that promotes replication in cyanobacteria and *Escherichia coli* (Taton *et al.*, 2014). Also the AmpR sequence was not detected by PCR. Although a negative PCR result is not very significant, it fits the other results. Taking the points above into account, it was concluded that no 7-K plasmid is present in the generated

Phormidium lacuna transformants. Furthermore, the results show that transformation of *Phormidium lacuna* was based on double recombination events. With single recombination event, the whole plasmid would be integrated into the genome and the PCR results in Figure 8 and Figure 20 would be different.

3.1.2 Protocol improvement

The establishment of the initial protocol is described under 3.1.1. This EP protocol I made three strains of *Phormidium lacuna* accessible for genetic manipulation, but it lacked high level efficiency (Figure 7). Also linked to low efficiency, but probably not exclusively, was the low reproducibility of the initial protocol: Conditions tested positive in one experiment did not result in transformants in another experiment (see also electronic supplement: transformation experiments). Besides low efficiency, low reproducibility presumably resulted from the fact that experiences in the handling of *Phormidium lacuna* were limited and its handling is more challenging than for other, frequently used model organisms. For further projects dependent on the transformation of *Phormidium lacuna*, a more efficient and reliable protocol was required and its development is outlined in this section.

3.1.2 a) Cell viability

Protocol optimization to improve efficiency and reproducibility of the transformation method started in autumn 2016. With start of the optimization procedure a strong loss in cell viability was observed in comparison to the phase of protocol establishment (early 2016): No cell propagation was observed on control plates without kanamycin in many cases and no cell propagation was observed on selective plates in all cases. The low cell viability in this time period could not be explained. Cell viability had to be restored to enable transformation of *Phormidium lacuna* again.

It was observed during the protocol establishment and preliminary experiments that washing steps and electroporation could affect cell viability. Restoration of cell viability was the crucial point for any further transformation experiments. Therefore, it was investigated in which way cell viability could be improved by different treatments. The effect of different factors on cell viability was monitored by the propagation of *Phormidium lacuna* on agar plates. Quantification of viable cells with counting chambers as Neubauer improved counting chamber (Brand, Germany) was not possible, since filaments do not arrange in one plane.

The impact of electroporation on cell viability has already been investigated before the establishment of the EP protocol I: Conditions were tested to setup the initial protocol under suitable conditions for *Phormidium lacuna* without the addition of DNA. Besides transfer and distribution of *Phormidium lacuna* on agar plates and suitable concentration of the selection marker kanamycin, also several electroporation parameters were tested. Negative effect of electroporation on cell viability could be observed for the lowest field strength tested (2.5 kV cm^{-1}, 2 ms) in comparison to cells that were not electroporated. With increasing field strength cell viability decreased further (tested until 15 kV cm^{-1},

2 ms). For the establishment of the EP protocol I 1 – 6 kV cm^{-1}, 4 ms were tested. Since transformants were generated in a range of 2.5 – 5 kV cm^{-1}, 3 – 4 ms, these conditions were considered a compromise between cell viability and a necessary treatment for transformation. The negative effect of electroporation on cell viability was therefore considered clarified.

In Figure 9 a loss of cell viability with increasing number of washing steps is shown. Cells plated on agar plates after the first centrifugation step showed stronger cell propagation than cells that underwent the complete washing procedure. This means that during washing procedure of the initial protocol viability of the cells decreased clearly. Combined with the subsequent electroporation, which decreased viability further, important factors for restoring cell viability are known.

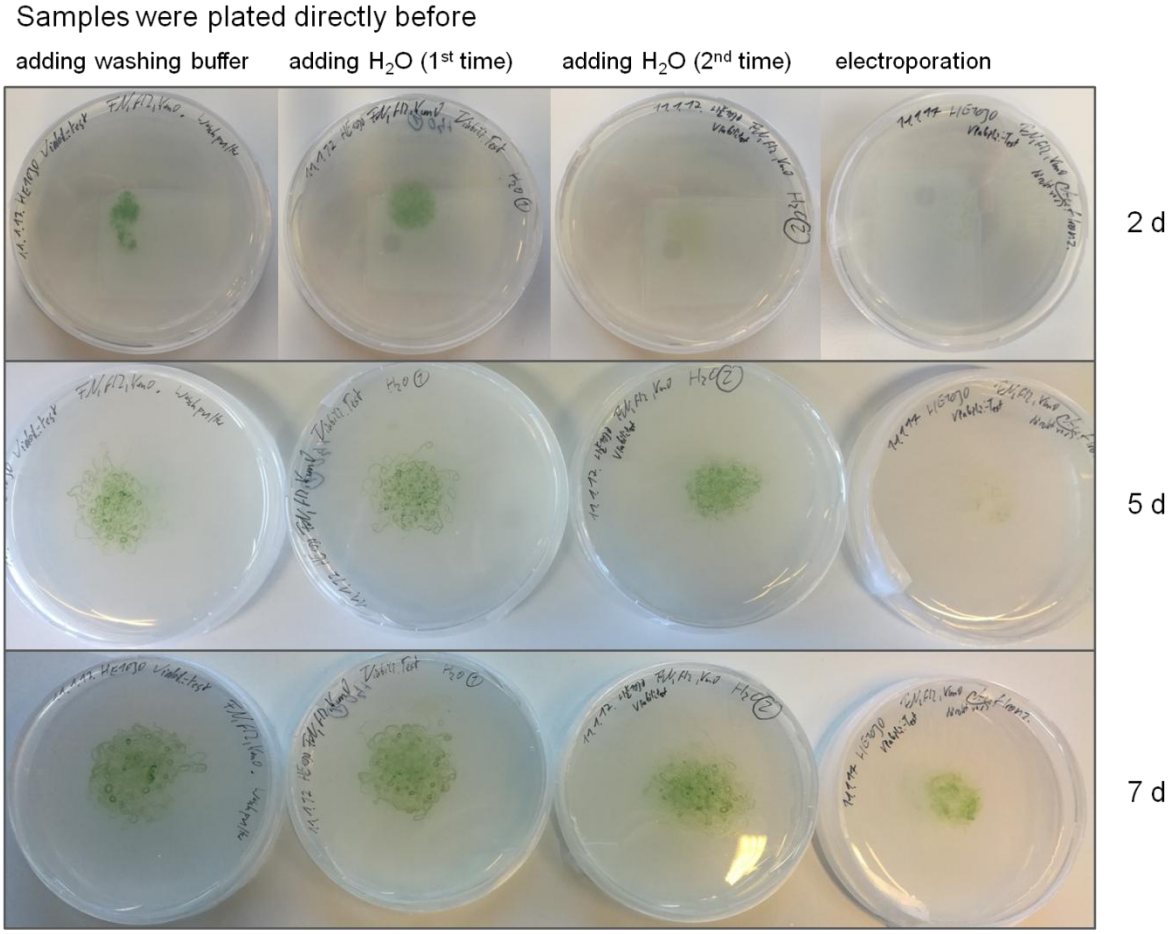


Figure 9: Propagation of *Phormidium lacuna* HE10JO samples on agar plates in dependency of washing steps at different time points. Samples were plated before the addition of washing buffer, before the first and the second washing step with water, and directly before electroporation. Cells were washed according EP protocol I.

It was tested if changes during the washing procedure could increase the cell viability. Cells were washed two times and concentrated. They were transferred on agar plates at different time points of this procedure. The effect of different cooling options was monitored by the resulting cell propagation as marker for cell viability. This is indicated in a semi-quantitative way (Table 8). During centrifugation all samples were cooled at 4 °C. Between the centrifugation steps samples were either

cooled in ice water, on crushed ice, or they were not cooled. Like already shown in Figure 9, the cell viability decreases during washing procedure but the rate of decrease is dependent on the cooling procedure between the centrifugation steps. The decrease of cell viability could be reduced when the cooling is optimized with ice water, while without any cooling the cell viability decreased further in comparison to cooling procedure of the initial protocol (crushed ice). In this experiment no cell propagation could be monitored at the end of the washing procedure when cells were cooled with crushed ice or not cooled at all (Table 8). Pictures illustrating this loss of cell viability can be found in the supplement (Figure 21).

Table 8: Propagation of *Phormidium lacuna* HE10JO samples on agar plates in dependency of the cooling procedure and the washing medium. Cells were washed two times and subsequently concentrated. Samples were plated at the indicated time points. Between the centrifugation steps cell were cooled in ice water, on crushed ice, or cells were not cooled. Cells were either washed with H_2O (tested for all cooling procedures) or they were washed with f/2 medium (cooling with crushed ice). The effect on cell propagation is an indicator for cell viability. Cell propagation is indicated by strong propagation in gradual differences (+++, ++, +), weak propagation (o), and no propagation (-).

	Cells were plated before		
Cooling procedure	adding H_2O (1^{st} time)	adding H_2O (2^{nd} time)	cell concentrating
ice water	+++	++	+
no cooling	+++	o	-
crushed ice	++	+	-
crushed ice (cells were washed with f/2 medium)	+++	+++	++

Also the effect of the washing medium was tested. The difference between washing with f/2 medium and H_2O (Table 8) illustrated that the use of H_2O as washing medium was the most critical point and not the centrifugation steps or other protocol related handling. Low osmolarity might be a reasonable explanation for this phenomenon. However, initial experiments with osmoprotectants like sorbitol and mannitol did not show beneficial effects. The effect of homogenization of *Phormidium lacuna* was investigated by Dominic Fink (bachelor thesis). It was shown that the standard protocol conditions of homogenization are well tolerated by *Phormidium lacuna* and thus should also not be a cause for dropping cell viability during the transformation protocol.

As consequence of the experiments shown in Figure 9 and Table 8, the amount of washing steps was reduced from three to two and cooling of the samples in ice water was adapted to the electroporation protocol. This restored the viability during EP so far that cells survived the protocol again. Two washing steps showed to be sufficient to remove salt from the cell supernatant and thus prevent sparks during electroporation due to remaining electrolytes.

3.1.2 b) Protocol optimization

Cell viability was restored with the changes mentioned in the sections above. In the resulting EP protocol II ice water was used for cooling and washing steps were reduced from three to two. The different changes during the protocol development can also be followed under 2.3.2. Further important changes between the transformation protocols are listed below.

However, the restoration of cell viability did not go in hand with a restoration of transformation efficiency. The EP protocol optimization with cultures of sufficient cell viability can be divided into two phases: Phase of low transformability – 13 experiments were performed (electronic supplement: transformation experiments). In only one approach of these experiments transformants were obtained. Phase of restored transformability – transformation efficiency was significantly higher than in previous phase due to the change of certain parameters (see below). With the restoration of transformability the effect of several factors on transformation efficiency could be tested. Thereby, the transformation procedure could be optimized stepwise from EP protocol II to the final EP protocol III.

It has to be mentioned that it was the aim of this work to establish a reliable and stable transformation protocol for *Phormidium lacuna*, since this was an essential requirement for any further, application orientated projects. For this objective, it was helpful but not mandatory to understand the influence of the single parameters in the protocol. It was therefore not the scope of this study to characterize the influence of every factor in the transformation protocol systematically. Furthermore, it would have been too extensive in respective of the required time and effort to monitor the mutual variables influencing the protocol development. In the subsequent sections the process of protocol optimization and the finding of natural competence in *Phormidium lacuna* are described.

3.1.2 c) Protocol optimization – Restoration of transformability

In the first time period of protocol optimization, only one successful transformation was performed. The first transformation attempt with the HE10DO strain during phase of low transformability was successful, but several attempts to repeat this under the same conditions failed. For GI09CO and HE10JO, no transformants were obtained at all during this phase. This single transformation event, however, showed that transformation in general was possible with EP protocol II.

The main focus was on *Phormidium lacuna* strains HE10JO and HE10DO while restoring transformability. HE10JO is the strain with the sequenced genome and first *Phormidium lacuna* strain to be transformed. HE10DO performed best in the bachelor thesis of Finn Zaiß in respect of transformation efficiency and could be transformed in the first trial after restored viability. Also, stock culture of the strain HE10DO is axenic in contrast to the stock culture of the strain HE10JO (see also 3.2).

Influenced by findings during restoration of cell viability, the EP protocol I was changed to the EP protocol II (see 2.3.2). Conditions, which were covered by the EP protocol II, and also additional

conditions were tested (electronic supplement: transformation experiments), but transformability with sufficient efficiency could not be restored at first.

Restoration on transformability in August 2017 correlated with two changes in the transformation procedure – the change of the microclimate during cultivation and the change of cell density. The microclimate of the cell shaker was changed by altering its position on the illumination rack and the air fluctuation. As a consequence, condensation events on the inner top of the culture flask could be omitted nearly completely. During the phase of protocol establishment no condensation events were observed in the culture flasks. The reason, why this changed, is not completely clear. However, no effect of condensation events of cell propagation was noticed. Condensation events inside the culture flask were therefore not considered critical at first. They are also not set in relation to the loss on cell viability mentioned above. However, transformability was restored when condensation events were omitted and situation regarding condensation events was comparable to the phase of protocol establishment. Influence of cell density on transformability is further characterized in the next section. The possible reasons, why these two changes (condensation events and cell density) resulted in restoration of transformability, are elucidated further in the discussion section (see 4.1.1).

The change of cultivation parameters only worked for the transformability of HE10DO. For the restoration of transformability of HE10JO and GI09CO more optimization steps have to be performed. Further work mainly focused on HE10DO. In direct comparison to previous experiments, restored transformability was achieved when the cell density of the HE10DO cultures was considerably higher (see Table 9 and electronic supplement: transformation experiments). The influence of cell shaker position was addressed in the experiment of 29.08.2017. Approaches were incubated in the old and new shaker position (electronic supplement: transformation experiments). But no approach resulted into transformants in this experiment.

Even though transformability was restored in general, some subsequent experiments did not result in any transformants. Thus, transformation efficiency had to be optimized further. The single optimization steps towards EP protocol III can be looked up in the electronic supplement (transformation experiments) and are examined in the next section.

Table 9: Transformation experiments of *Phormidium lacuna* HE10DO directly before and after the restoration of transformability in 2017. Experiments with *Phormidium lacuna* HE10JO, incubation time, and additional remarks of experimental conditions are not shown and can be looked up in the electronic supplement (transformation experiments). 100 ml culture volume was used in all shown experiments. Washing buffer: (2 mM tricine, 2 mM EDTA, pH 8). a: only one washing step. Approaches, which resulted in transformants, are indicated with "+". Approaches without transformants are indicated with "-".

Date	Washing medium	OD_{750}	plasmid, name	plasmid, amount [µg]	Field strength, pulse duration	Transformation
04.07.	0.2 mM EDTA	0.09	7-K	15	3 kV cm^{-1}, 4 ms	-
	0.2 mM EDTA		7-K	15	3 kV cm^{-1}, 4 ms	-
	water		7-K	15	3 kV cm^{-1}, 4 ms	-
	water		7-K	15	3 kV cm^{-1}, 4 ms	-
03.08.	0.35 mM EDTA	0.08	7-K	30	3 kV cm^{-1}, 4 ms	-
	0.35 mM EDTA		7-K	30	5 kV cm^{-1}, 4 ms	-
	water		7-K	30	3 kV cm^{-1}, 4 ms	-
	water		7-K	30	5 kV cm^{-1}, 4 ms	-
Changed microclimate of the incubation shaker: Prevention of condensation events in cell culture flasks.						
12.08.	0.5 mM EDTA	0.28	7-K	15	3 kV cm^{-1}, 4 ms	+
	0.5 mM EDTA		7-K	15	5 kV cm^{-1}, 4 ms	-
15.08.	0.8 mM EDTA	0.28	7-K	15	3 kV cm^{-1}, 4 ms	+
	0.8 mM EDTA		7-K	15	5 kV cm^{-1}, 4 ms	-
	0.8 mM EDTA		7-K	15	7 kV cm^{-1}, 4 ms	-
	0.8 mM EDTA		7-K	15	3 kV cm^{-1}, 4 ms	-
	0.8 mM EDTA		7-K	15	5 kV cm^{-1}, 4 ms	-
	0.8 mM EDTA		7-K	15	7 kV cm^{-1}, 4 ms	-
29.08.	washing buffer	0.37	7-K	15	4 kV cm^{-1}, 4 ms	-
	washing buffer		7-GFP_Kn	15	4 kV cm^{-1}, 4 ms	-
	washing buffer (a)		7-K	15	4 kV cm^{-1}, 4 ms	-
	washing buffer (a)		7-GFP-pKn	15	4 kV cm^{-1}, 4 ms	-
	0.8 mM EDTA		7-K	15	4 kV cm^{-1}, 4 ms	-
	0.8 mM EDTA		7-GFP_Kn	15	4 kV cm^{-1}, 4 ms	-
	0.8 mM EDTA		7-K	15	4 kV cm^{-1}, 4 ms	-
	0.8 mM EDTA		7-GFP_Kn	15	4 kV cm^{-1}, 4 ms	-
	0.8 mM EDTA		7-K	15	4 kV cm^{-1}, 4 ms	-
	0.8 mM EDTA		7-GFP_Kn	15	4 kV cm^{-1}, 4 ms	-
06.09.	water	0.31	7-K	15	3 kV cm^{-1}, 4 ms	-
	water		7-K	15	3 kV cm^{-1}, 4 ms	+
	0.6 mM EDTA		7-K	15	3 kV cm^{-1}, 4 ms	+
	0.6 mM EDTA		7-K	15	3 kV cm^{-1}, 4 ms	+
	0.6 mM EDTA		7-K	30	3 kV cm^{-1}, 4 ms	-
	0.6 mM EDTA		7-K	15	3 kV cm^{-1}, 4 ms	-
04.10.	0.6 mM EDTA	0.31	7-K	19	3 kV cm^{-1}, 4 ms	+
	0.6 mM EDTA		7-K	30	3 kV cm^{-1}, 4 ms	-
	0.6 mM EDTA		7-K	30	3 kV cm^{-1}, 4 ms	+
	0.6 mM EDTA		7-K	15	3 kV cm^{-1}, 4 ms	+
	0.6 mM EDTA		7-K	30	3 kV cm^{-1}, 4 ms	+
	0.6 mM EDTA		55-K	30	3 kV cm^{-1}, 4 ms	-
	0.6 mM EDTA		2-K	30	3 kV cm^{-1}, 4 ms	-

3.1.2 d) Protocol optimization – factors

In the following, the impact of particular factors on transformation efficiency is considered. The data set allowed an overall comparison of transformation efficiencies in dependency of the respective transformation protocol (Figure 12 in 3.1.3). The major factors tested and their potential influence is examined below. The electronic supplement can be considered for the single experiments and all factors tested during protocol optimization (see transformation experiments).

Three central points were regarded to be critical to restore transformability and increase protocol efficiency: **Cell viability** for high survival rates, **DNA uptake** into the cell, the **homologous recombination rate**.

Additionally, the handling with *Phormidium lacuna* was optimized further. The filamentous growth of *Phormidium lacuna* and its strong tendency to form aggregates was a continuous challenge. Homogenization, precise pipetting, and frequent inversion of cell suspension were initial strategies that allowed comparative treatment of samples. The comparability was further improved during the optimization towards EP protocol III by inoculation of bacterial cultures to a calculated start OD_{750} (0.001 – 0.003). Thereby, desired cell density was normally reached in 6 d. For optimal cell density during transformation see below.

Initially, transformants were detected if their propagation on agar plates is observable by eye. The detection of transformants was improved by the use of microscopy. First, transformants could be identified earlier. Transformed and untransformed cells were discriminated by color and filament shape on selective agar plates. Filaments of transformed cells are long, have a smooth shape and intensive green color like *Phormidium lacuna* WT without selective pressure. Under selective pressure, filaments of untransformed cells break over time at various points and thus get continuously shorter and fragmented. Also their shape is getting uneven and the pigmentation is turning from green into green-yellow. Thus, transformed filaments could be identified with microscopy and transferred into selective suspension culture before untransformed cells died. Time required for the detection of transformants was thereby reduced and transformants could be detected after one or two weeks. In Figure 10 representative examples of different stages during selection are shown.

Detection of transformants by microscopy has an additional advantage. Higher kanamycin concentration (120 µg/ml) can also arrest cell propagation of transformants on agar plates, which will subsequently die. Transfer into selective suspension culture in time restored cell propagation of growth-arrested transformants. Growth-arrested transformants were often only detectable by microscopy and would have been overseen without microscopy. The reason why propagation arrest of transformants was observed on agar plates but not in suspension culture was not addressed experimentally. It seems that cells are more vulnerable to kanamycin on agar plates than in suspension culture (see also discussion under 4.1.2).

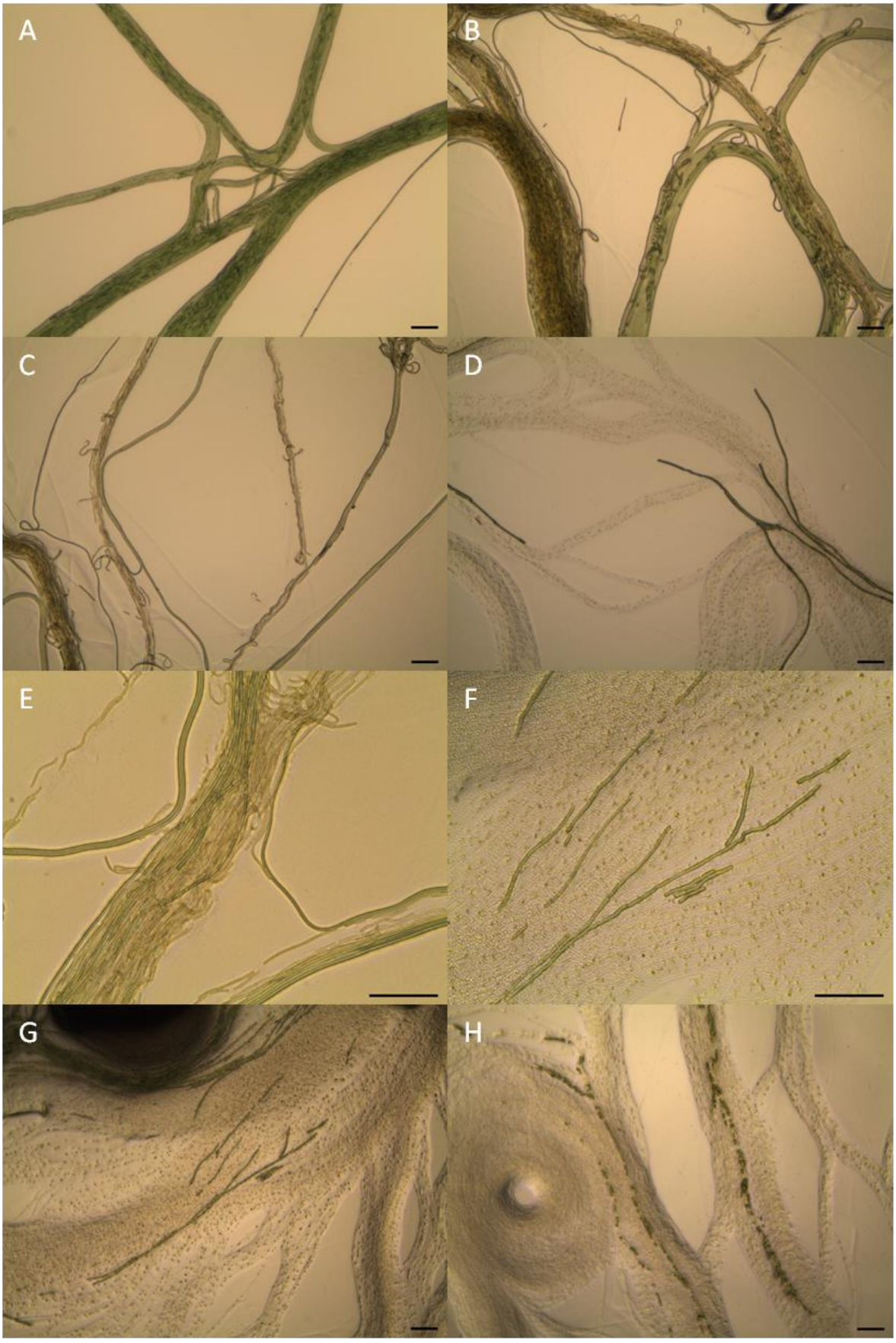


Figure 10: Light microscopy exemplary images of HE10DO on agar plates (f/2) in different stages of selection. For description see next page.

Figure 10 (continued): Light microscopy exemplary images of HE10DO on agar plates (f/2) in different stages of selection. Light microscopy, bright field, magnification: 40x or 100x, scale bars indicates 100 µm. A) Advanced growth of transformant (120 µg/ml kanamycin). Picture is representative for unrestricted propagation of HE10DO WT and HE10DO transformants. B and C) Transformed cells and WT cells on selective plates (70 µg/ml kanamycin) in early and later phase of selection, respectively. Transformed and WT cells could be distinguished by their color. WT cells on selective plates turned green-yellow. The change of pigmentation is an indication for stress and correlates with the dying of the cells. Transformed cells stayed green. D) Transformed cells after untransformed cells have died (120 µg/ml kanamycin). E) Picture of late stage of selection (70 µg/ml kanamycin) in higher magnification. Transformed and WT cells could be differentiated by color. F, G, and H) Higher kanamycin concentration (120 µg/ml) could also arrest propagation of transformants on agar plates: F+G) Middle stage of growth arrest in two magnifications. H) Late stage of growth arrest.

Even though **cell viability** was restored to a level that allowed further protocol optimization, it was still a crucial point that needs further optimization. Some experiments during protocol optimization were not evaluable due to an interim drop of cell viability (electronic supplement: transformation experiments).

Homogenization with Ultraturrax (10,000 rpm, 3 min) is a rather harsh mechanical treatment but it was not considered as harmful for *Phormidium lacuna*. It is demonstrated in Table 8 and Figure 9 that after cell suspension was homogenized cell viability is still very high. It was also demonstrated by Dominic Fink (bachelor thesis) that *Phormidium lacuna* can tolerate homogenization by the Ultraturrax very well. It was hypothesized that during the washing procedure the vulnerability of *Phormidium lacuna* towards mechanical stresses might increase since also the overall cell viability decreases. Also a change of cell aggregation can be observed during the washing procedure that might be linked to the loss of cell viability. Therefore, it was tried to reduce mechanical stresses, especially during later washing steps and the electric pulse with the use of cut pipette tips and a reduced pipetting speed. Also, cuvettes were flushed with medium immediately after EP to transfer cells as soon as possible into the preferred environment.

During the phase of low transformability the only transformation event is reported for a lower cell density (OD_{750} = 0.12). This supported the misleading assumption that a lower cell density is beneficial for transformation. In contrast, the restoration of transformability is correlated with an increase of the OD_{750} (Table 9 and electronic supplement: transformation experiments). Therefore, the OD_{750} range for transformation was changed to 0.2 – 0.4 and later to 0.25 – 0.35 in EP protocol III. This change most likely promoted further transformation events. It was hypothesized at this point that with higher cell density cell viability after electroporation increases on the one hand because there are simply more cells used during the procedure. On the other hand cells in later growth phase might tolerate electroporation better. For the effect of cell density on transformation also consider the discussion section (4.1.1). Also the applied field strength was changed to 3 kV cm^{-1}, because all transformants after the restoration of transformability were generated with this parameter (Table 9). The negative effect of EP on cell viability is already mentioned above. Considering only experiment with the OD_{750} range of 0.25 – 0.35 and field strength of 3 kV cm^{-1} (electronic supplement: transformation experiments), every transformation experiment from August 2017 on resulted in

transformants with at least one tested condition. This underlines the influence of cell viability on transformation efficiency. Two additional factors that probably influenced cell viability were the incubation time on ice prior to EP and DNA purity. Please consider therefore the explanatory text to Table 10 and 3.1.3.

The effect of EDTA on transformation was considered ambivalent, possibly increasing transformability on the one hand, but also definitely decreasing cell viability. Especially for the strain HE10DO a negative effect on cell viability was observable. This was first described by Finn Zaiß (bachelor thesis) and was also observed in this study. If the strain HE10DO is incubated in washing buffer (2 mM tricine, 2 mM EDTA, pH 8; EP protocol I), the color of the cell suspension turns yellow in a time dependant manner. This effect was stronger at higher concentrations of EDTA. The change of color of *Phormidium lacuna* could be also observed for WT cells under selective pressure or after prolonged cultivation of *Phormidium lacuna* cultures. A change of pigmentation towards yellow is therefore generally correlated with dying of *Phormidium lacuna*. Therefore, the washing buffer in the first washing step in the EP protocol I was changed to EDTA solution of lower concentration. Also it was tested in parallel to use only water in all washing steps. With reduced EDTA concentration, HE10DO cultures did not show pigmentation change during the first washing step. Since EDTA was part of the washing buffer in the EP protocol I, which initially enabled the transformation of *Phormidium lacuna*, it was also hypothesized that EDTA might be beneficial or required for the transformation of *Phormidium lacuna*. Therefore, different EDTA concentrations were tested to find a balance concentration between harmful and beneficial amount of EDTA (similar to the parameters of electroporation).

Concerning **DNA uptake**, EDTA was considered potentially beneficial, because of its ability to complex divalent cations. Thereby, EDTA can decrease the stability of the cell wall itself as well as other extracellular structures like exopolysaccharides (Gray & Wilkinson, 1965, Alakomi *et al.*, 2006). The inhibition of extracellular nucleases could be a beneficial effect as well (Cao *et al.*, 1999). Both effects may increase transformability of *Phormidium lacuna* by either increasing the permeability induced by electroporation or inhibiting nucleases. Nucleases are a factor, which is often interfering with the transformation of cyanobacteria (Takahashi *et al.*, 1996, Stucken *et al.*, 2013). However, it was hard to monitor a potentially beneficial effect of EDTA in single experiments precisely. In an overall comparison of many experiments, the effect of water and EDTA as washing medium was characterized. In Figure 11 the transformation efficiencies of all EP approaches of various experiments with conditions of EP protocol III (but with 7-K plasmid) are shown in respect of the medium of the first washing step. This revealed that there is no distinct differences influence on transformation efficiency by EDTA solution and water. In the overall comparison also fluctuations of the experiments are included. Experimental settings, cell viability, and efficiency of gene transfer varied. Therefore, the effect of EDTA on transformation was monitored additionally in three subsequent experiments under the same conditions (Table 10). In this direct comparison, only approaches washed with water in

the first washing step resulted in transformants. Samples washed with 0.6 mM or 1.2 mM EDTA solution, respectively, in the first washing step did not result in any transformants. Since there was not monitored a positive effect in the overall comparison (Figure 11) and in the direct comparison (Table 10), EDTA solution was replaced by H_2O in the first washing step of the transformation protocols (EP protocol III and NT protocol).

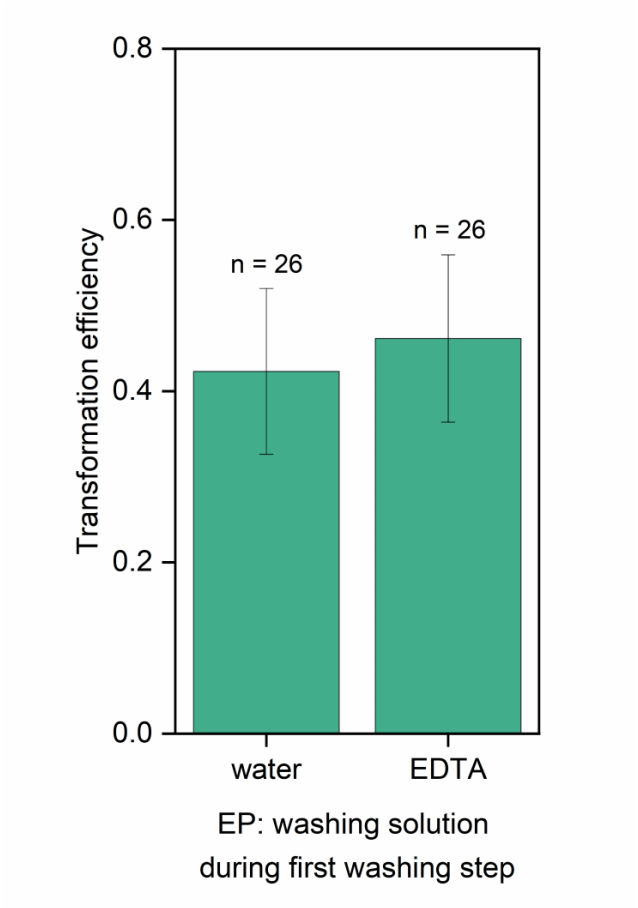


Figure 11: Transformation efficiency of *Phormidium lacuna* HE10DO during electroporation (EP) in dependency of the washing solution during the first washing step. Cells were either washed with water or an EDTA solution (pH 8, various concentrations) in the first washing step. Number of monitored experiments is indicated. Error bars show standard error. Significance: T-test error probabilities are above threshold (5%).

To increase the DNA uptake the field strength was varied in several transformation experiments before and after the restoration of transformability. Field strengths up to 8 kV cm^{-1} were tested (for detailed information consider electronic supplement: transformation experiments). But in 2017, only approaches with 3 kV cm^{-1} resulted into transformants (see also above, Table 9, and electronic supplement: transformation experiments). Therefore, it was concluded that DNA uptake cannot be improved by field strengths above 3 kV cm^{-1}. For EP mediated DNA uptake also consider 3.1.3 and 4.1.1.

Another strategy to optimize the DNA uptake was to increase the DNA amount in the transformation approaches. DNA amounts of 30 µg and 15 µg per transformation approach were tested frequently. To monitor the minimal amount of plasmid DNA necessary for transformation also lower amounts were

used. Plasmid DNA amounts from 1 µg to 5 µg per approach were grouped together for an overall analysis. For single experiments concerning DNA amount consider the electronic supplement (transformation experiments). The overall comparison revealed that the monitored amounts of plasmid DNA did not cause different transformation efficiencies (green bars in Figure 15). The slightly higher transformation efficiency for 15 µg plasmid is not significant.

The third point considered in protocol optimization is the **rate of homologous recombination**. It was unclear if homologous recombination is a rate limiting state during the transformation of *Phormidium lacuna*. Therefore, transformation of *Phormidium lacuna* was tested with a self-replicating plasmid in cyanobacteria. Thus, homologous recombination is not required for transformation. The plasmid pVZ321 has a replicon from the RSF1010 plasmid that allows replication in a broad range of gram negative bacteria including cyanobacteria (Zinchenko *et al.*, 1999). This approach did not result in transformants. However, the experiments of this approach were performed in phase of low transformability and during an intermediate loss of viability (October 2017, see also electronic supplement: transformation experiments). Thus, it cannot be concluded that transformation with this plasmid is generally not possible in *Phormidium lacuna*.

The integration rate of heterologous sequence by homologous recombination is influenced by the length on the flanking sequences. This was also reported for cyanobacteria, namely *Synechocystis* sp. PCC 6803, for which plasmid with longer homologous sequences showed also higher transformation rates (Zang *et al.*, 2007). Therefore, the length of the flanking sequences was doubled from ca. 500 to ca. 1000 bp on each side to increase rate of homologous recombination. The longer homologous sequences are indicated with the affix ":2k", for example 7-K:2k.

In three subsequent experiments with comparable condition, the impact of increased homologous sequence length was tested with the transformation of 7-K and 7-K:2k, respectively (Table 10). Also the influence of the washing agent in the first washing step was investigated (see Table 10 and text above). Only the plasmid with elongated, homologous sequences resulted in transformants in EP experiments. This indicates clearly that a longer homologous sequence is beneficial for transformation. This is explainable with a higher efficiency of homologous recombination.

Table 10: Transformation of HE10DO in dependency of homologous sequence length and EDTA on HE10DO transformation. The results of three independent transformation experiments are shown. Approaches, which resulted in transformants, are indicated with "+". Approaches without transformants are indicated with "-". In the third experiment, natural transformation is demonstrated the first time for *Phormidium lacuna*.

Plasmid	1. washing step	EP parameter	06.12.2017	13.12.2017	20.12.2017
7-K	H_2O	3 kV cm^{-1}, 4 ms	-	-	-
7-K:2k	H_2O	3 kV cm^{-1}, 4 ms	+	+	+
7-K	0.6 mM EDTA	3 kV cm^{-1}, 4 ms	-	-	-
7-K:2k	0.6 mM EDTA	3 kV cm^{-1}, 4 ms	-	-	-
7-K	1.2 mM EDTA	3 kV cm^{-1}, 4 ms	-	-	not tested
7-K:2k	1.2 mM EDTA	3 kV cm^{-1}, 4 ms	-	-	not tested
7-K	H_2O	no EP	not tested		+

It was already mentioned above that cell viability was still a critical point during protocol optimization. In the experiments in Table 10 the cell viability was reduced, which was probably related with interim problems with plasmid purity after preparation. It was observed that cell propagation on agar plates was considerably lower in samples with plasmid DNA (transformation approach) than in the respective samples without plasmid DNA (negative control). This might explain why approaches with the plasmid 7-K did not result in transformants in these experiments. Cell viability is also reduced by EP (see also above). In the third experiment in Table 10 for one sample the EP step was skipped to monitor the negative effect of plasmid DNA alone. Indeed, cell viability was considerably higher without EP, also in comparison to samples without plasmid DNA (negative controls for EP experiments). This showed that the negative effect of plasmid DNA was negligible without EP.

Surprisingly, this approach without EP also resulted in transformants, even though the plasmid with shorter homologous sequences, 7-K, was used. This means that *Phormidium lacuna* is also naturally transformable with the final protocol just by skipping the EP step. The natural transformation of *Phormidium lacuna* is further characterized in the next section.

3.1.3 Natural transformation

The finding of natural transformation (NT) in *Phormidium lacuna* was unexpected, since it was once tested negative for HE10JO in 2016 with a variation of the EP protocol I (electronic supplement: transformation experiments). Also in literature NT in commonly described as an exclusive trait of a small group of unicellular organisms among the cyanobacteria (see also 1.3). This is the first report for NT in the order Oscillatoriales. In respect of heterologous gene expression this is the first demonstration of NT for all filamentous cyanobacteria (mutant complementation was shown once for one species of the genus *Nostoc* in the early 80s (Trehan & Sinha, 1982)). An immediately uprising question was whether we have two distinct transformation techniques, EP and NT – or if NT was permitting transformation also during EP protocols. In this section NT is considered in respect of development of the transformation protocol of *Phormidium lacuna*. In section 3.4, the finding of NT in *Phormidium lacuna* is set in relation to other cyanobacteria.

3.1.3 a) Characterization of natural transformation

In the first experimental proof of NT in *Phormidium lacuna* (Table 10), it was not only shown that EP is not necessary for transformation of *Phormidium lacuna* HE10DO. It was also indicated that NT is potentially more efficient. The plasmid with shorter homologous sequences (7-K) only resulted in transformants in the NT approach but not in any EP approach.

The higher efficiency of NT in comparison to EP is also demonstrated in an overall comparison of the different protocol's transformation efficiencies (Figure 12). The transformation efficiency for HE10JO with the EP protocol I was ca. 40%. The transformability of HE10JO could not be reconstituted with

the EP protocol II, EP protocol III, and the NT protocol after the loss of cell viability in late 2016. For HE10DO, only one out of 20 transformation attempts resulted in transformants with EP protocol II. With EP protocol III transformability could be reestablished for HE10DO to a comparable level like for HE10JO with the EP protocol I. Transformation efficiency with the protocol for NT was ca. 90% (strain HE10DO) and thus significantly higher than with EP. The only relevant difference between the EP protocol III and the NT protocol was that the electric pulse was skipped.

In the analysis of this section all conditions leading to transformants with EP since the restoration of transformability are included in the data set for EP protocol III. This includes also the use of EDTA solutions in the first washing step. In the methods section this treatment was excluded from the protocol description since it was shown in 3.1.2 that no beneficial effect on transformation is detectable.

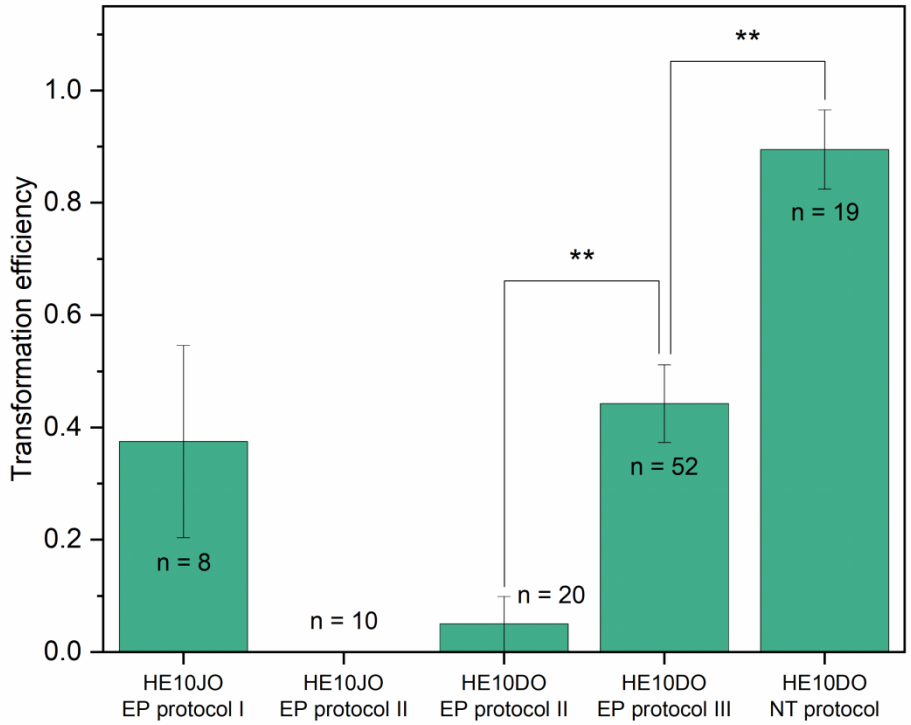


Figure 12: Transformation efficiency of *Phormidium lacuna* for different steps during protocol development. Transformation efficiencies are shown for the electroporation protocols I, II, and III and for the natural transformation protocol for the strains HE10JO and HE10DO. Number of monitored approaches from different experiments is indicated. Error bars show standard error. Significance: T-test error probabilities p for transformation efficiency are indicated by *, $p < 5\%$ and **, $p < 1\%$. Only plasmids were used in this analysis, which have only the *kanR* operon as insert (7-K, 7-Kn, 7-K:2k, 7-Kn:2k).

The 90% natural transformation efficiency was shown for plasmid, which have only the *kanR* operon as insert. The constructs for ethanol production (encoding for PDC alone or PDC and ADH) were all transformed with the NT protocol, but with declined transformation efficiency (Figure 13; PDC: pyruvate decarboxylase, ADH: alcohol dehydrogenase). With increasing insert size (*kanR* operon: ca. 1200 bp, *pdc* and *kanR*: ca. 3000 bp, *pdc*, *adh* and *kanR*: ca. 4200 bp) transformation efficiency

decreased: Plasmids with the *pdc* and *kanR* insert (7-P-K:2k and 7-pP_K:2k) had a transformation efficiency of ca. 65% or ca. 50%, respectively. The plasmid carrying additionally ADH had even lower transformation efficiency. It cannot be stated if the differences in transformation efficiencies in Figure 13 are significant due to the small data set for the plasmids for ethanol production (T test error is always above threshold of 5%). However, the correlation of decreasing transformation efficiency with increasing insert size is also observed by Nora Weber in her master thesis (preliminary data).

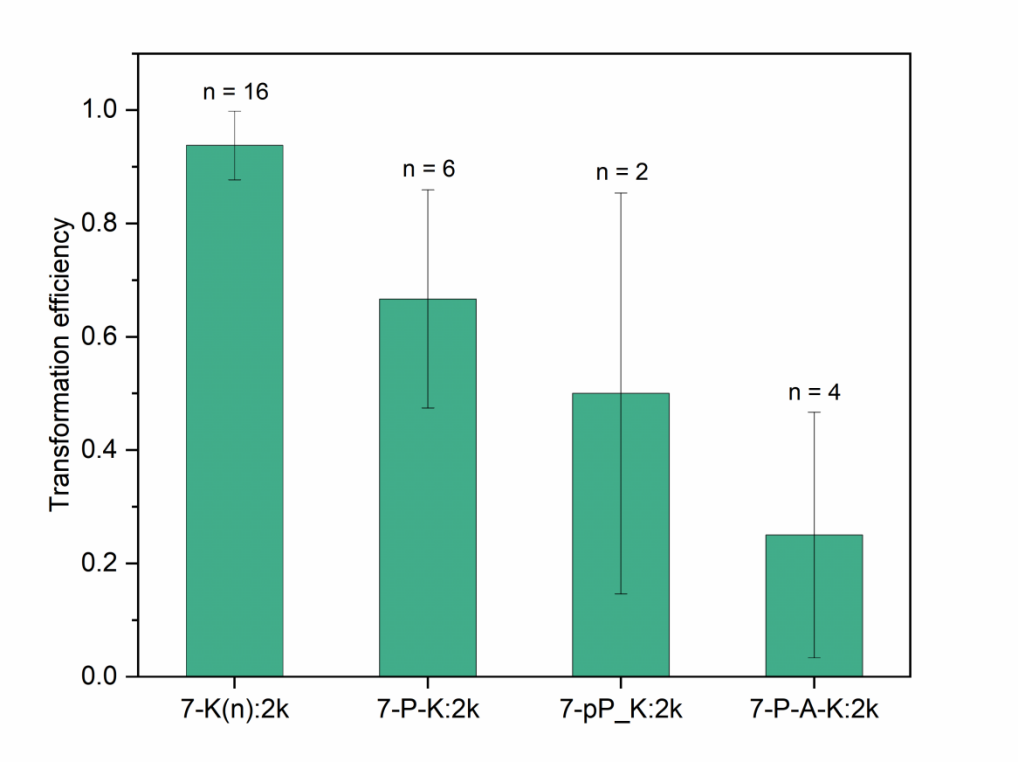


Figure 13: Transformation efficiency of *Phormidium lacuna* HE10DO dependent on insert size for the natural transformation protocol. Insert sizes: 7-K(n):2k – 1347 bp (7-K:2k) or 1216 bp (7-Kn:2k). 7-P-K:2k – 3073 bp. 7-pP_K:2k – 3322 bp. 7-P-A-K:2k – 4241 bp. Number of monitored experiments is indicated. Error bars show standard error. Significance: T-test error probabilities above threshold (5%).

For the transformation of *Phormidium lacuna* two version of the *kanR* operon were used, KanR (K) and KanRn (Kn). *Phormidium lacuna* transformants of the two different operon versions did not differ in phenotype concerning cell viability, propagation, or resistance towards kanamycin. Therefore, it was not distinguished between these two operon versions in the overall analyses. If both versions are included in an analysis, this is indicated by K(n). For the difference of 7-K and 7-Kn or 7-K:2k and 7-Kn:2k please consider 2.1.2.

In Figure 14, transformation efficiency of the EP and NT method are compared with each other, also in respect of the length of the flanking sequences. The green bars indicate the transformation efficiency of both methods either in all tested conditions since restoration of transformability or under final conditions (EP protocol III or NT protocol). For NT, all experiments were performed under final conditions. Transformation efficiency was ca. 90% for NT and ca. 30% and 40% for EP, respectively. No influence of the homologous sequence length could be documented for EP (patterned blue and

grey bars). For NT, 7-K(n) (e.g. ca. 500 bp flanking sequence) seemed to have lower efficiency, but the low number of data for 7-K interfered with a precise characterization of transformation efficiency in this case. In Table 10 it is shown that longer homologous sequences enhance transformability during EP. A comparison between Table 10 and Figure 14 is further addressed in the discussion section.

For NT homogenization is sufficient. This was tested in one experiment without subsequent washing steps. In this experiment two approaches with 30 µg or 3 µg of the plasmid 7-Kn:2k resulted both in transformants. This finding indicated that the washing procedure was not essential for the NT protocol, but it might have a supportive effect (see also 4.1.1).

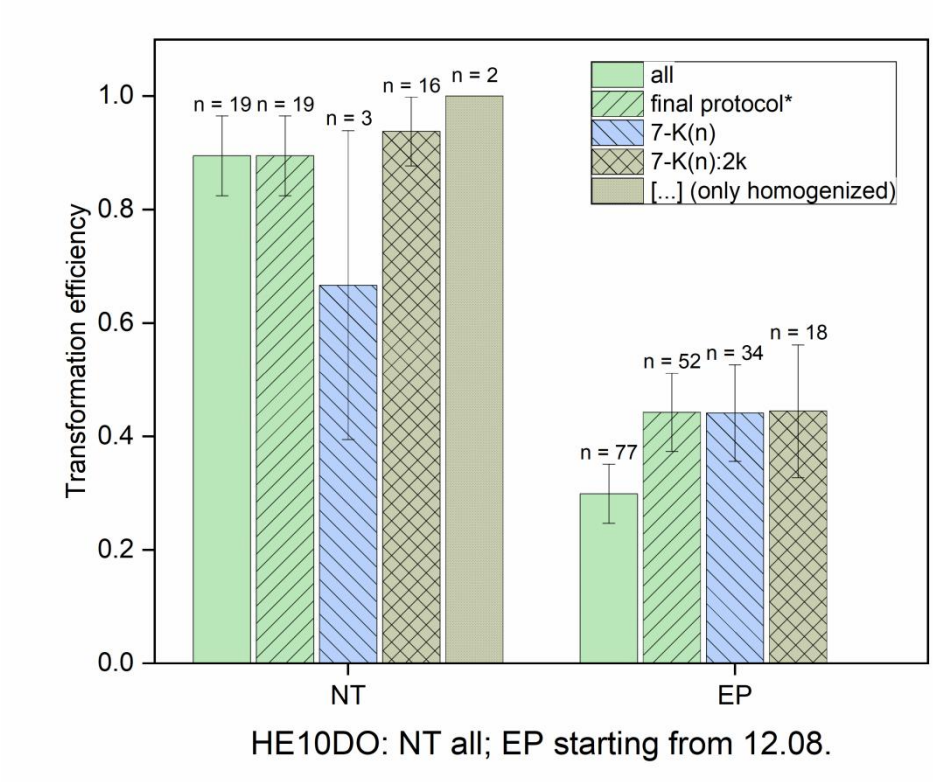


Figure 14: Transformation efficiency of *Phormidium lacuna* HE10DO in dependency of the transformations procedures NT and EP. Green bar, blank: All approaches with 7-K(n) and 7-K(n):2k. Green bar, patterned: All approaches with final protocol conditions (blue and grey bar patterned combined). Blue bar, patterned: Approaches with plasmid 7-K(n). Grey bar, patterned: Approaches with plasmid 7-K(n):2k. Grey bar, blank: Approaches with plasmid 7-K(n):2k – cells were only homogenized and DNA was added (no washing steps). Number of monitored experiments is indicated. Error bars show standard error. Significance: T-test error probabilities between EP and NT are indicated in Figure 12. T-test error probabilities of the bars of NT and EP among each other are above threshold (5%). NT: natural transformation. EP: electroporation.

The effect of plasmid DNA amount on transformation efficiency on NT and EP for plasmids with only *kanR* operon insert is shown in Figure 15. No significant difference could be found in the range between 1 – 30 µg among the green or blue bars, respectively. Experiments with 1 – 5 µg plasmid were grouped together because otherwise the number of compared experiment would have been too low. 30 µg plasmid and 15 µg plasmid were tested more often and the respective transformation

efficiency can be monitored alone. Below 1 µg only one positive transformation event was once monitored for EP, indicating that the critical amount of plasmid for transformation was somewhere below 1 µg plasmid. An amount of 3 – 5 µg plasmid is thus a reasonable amount to achieve maximal transformation efficiency without wasting plasmid. A reasonable explanation of the lower transformation efficiency of the EP protocols is the negative effect of EP on cell viability. If EP would have a beneficial effect on transformation of HE10DO, this would be hard to monitor due to this negative effect. One possible way, in which EP could promote transformation, is that lower amount of plasmid is required to generate transformants. The minimum amount of DNA with effective transformation was 0.3 µg for the EP protocol, while it was 3 µg for the NT protocol. These data are based on single experiments, however (see also electronic supplement: transformation experiments).

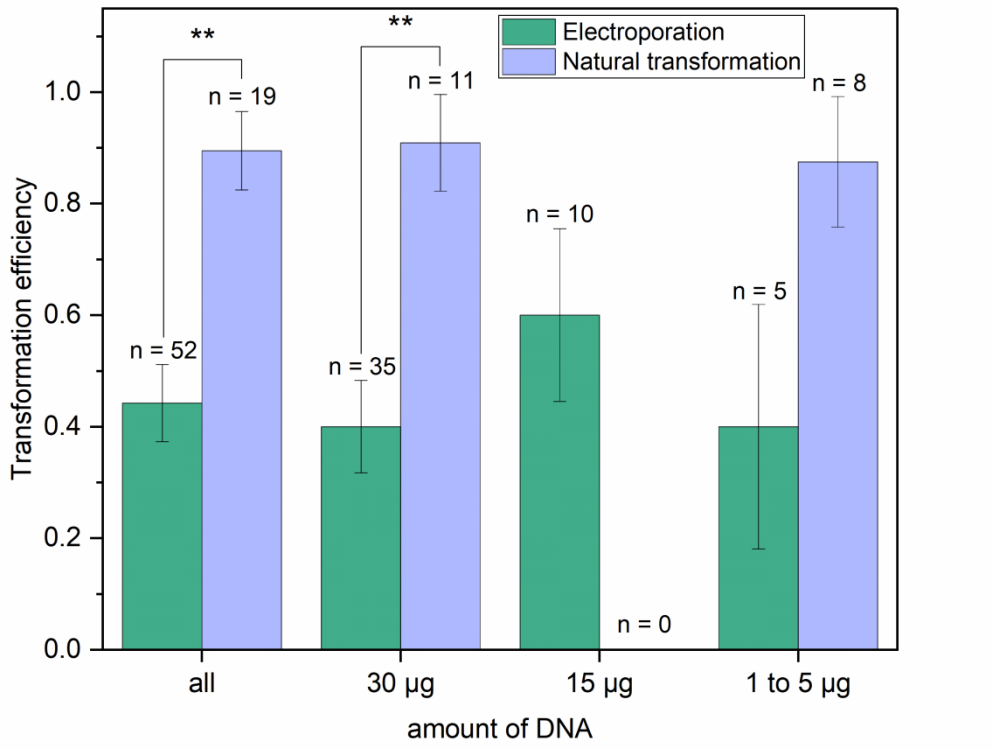


Figure 15: Transformation efficiency of *Phormidium lacuna* HE10DO during EP and NT in dependency of the DNA amount. Transformation experiments with plasmids were considered, in which only the *kanR* operon was used as insert. The DNA amount of 15µg was only tested for EP. Number of monitored experiments is indicated. Error bars show standard error. Significance: T-test error probabilities p for transformation efficiency are indicated by *, p < 5% and **, p < 1%. T-test error probabilities are above threshold (5%) for 1 to 5 µg and for NT and EP among each other, respectively. NT: natural transformation. EP: electroporation.

3.1.3 b) Time point of natural transformation

In the question if EP has a positive effect on transformation of *Phormidium lacuna* at all or if all transformants go back on NT, the time point of NT is an important factor. Therefore, plasmid was added at different points of the NT procedure (Table 11): For two approaches the plasmid was added

as usual to the concentrated cells. One sample was the positive control for standard transformation conditions. The other sample was washed after an incubation time of 15 min two times with water (analog to the washing after homogenization of the suspension culture) in order to remove free plasmid again. For three approaches DNA was added after the cells were transferred to the suspension culture: To one sample the plasmid was added immediately after the cells were transferred in suspension culture. For the other two samples DNA was added after 12 h or 18 h, respectively.

Table 11: Time point of natural transformation. Plasmid DNA was added to concentrated cells or to suspension culture of *Phormidium lacuna* HE10DO at different time points. Transformation was not possible when plasmid DNA was removed by subsequent washing steps. Approaches, which resulted in transformants, are indicated with "+". Approaches without transformants are indicated with "-".

Addition of DNA		Incubation time on ice	Selection
to concentrated cells	standard procedure	15 min (with plasmid)	+
	additional washing steps (2x) to remove DNA in the supernatant		-
to suspension culture	direct	15 min (without plasmid)	+
	after 12 h		+
	after 18 h		+

The only sample that could not be transformed was the one, in which supernatant containing the plasmid was removed with two washing steps. This demonstrates that the moment of NT is during the two days cultivation in suspension culture or maybe even on the agar plate. If transformation takes place during suspension culture, not even the complete two days of cultivation are necessary for this. The additional two washing steps seemed to be enough for removing the DNA, so no transformation was possible is suspension culture. It also shows that DNA uptake did not happen while the concentrated cells were incubated on ice with the plasmid. It was demonstrated above that additional washing steps can reduce cell viability drastically, so it cannot be excluded completely that no transformants were obtained due to increased stress. However, reduced cell viability for this sample could not be observed in this experiment.

Since the time point of NT is during the two days cultivation in suspension culture or on agar plate and since the only difference between EP protocol III and the NT protocol is the electric pulse, it is also possible that EP approaches were transformed by NT. Also no clear experimental proof could be found so far that EP enhances transformation in comparison to NT. In contrast, transformation efficiency is much lower with EP. So, there is no evidence that EP contributes to transformation in *Phormidium lacuna*.

Incubation of concentrated cell suspension and DNA on ice was performed is several approaches. Until NT of *Phormidium lacuna* was discovered, all samples during transformation procedure were first mixed with DNA and then pulsed in the same order. This results in an average incubation on ice between 10 – 20 min. In several experiments also prolonged incubation of ice was tested (electronic supplement: transformation experiments). This procedure was changed with the finding of NT. All samples for EP were mixed with DNA and immediately electroporated, while samples for NT were

incubated on ice. Since the experiment (Table 11) show that DNA can be taken up during the two days incubation in suspension culture, it is demonstrated that incubation on ice is not necessary during the transformation protocol. It is rather disadvantageous, since prolonged incubation in water can decrease viability, which was demonstrated during the washing procedure (Figure 9). Therefore, incubation of DNA and concentrated cells on ice after the washing procedure was excluded from the final transformation protocols.

3.1.4. Summary of protocol development

Transformation protocol development for *Phormidium lacuna* resulted in the finding of NT in a new order of the cyanobacteria. An efficient NT protocol via homologous recombination in the *Phormidium lacuna* strain HE10DO was established (~ 90% transformation efficiency). Thereby, *Phormidium lacuna* is accessible for genetic manipulation and recombinant biotechnology.

Time point of NT and also the significantly higher efficiency indicate that all transformation events can be probably derived from NT. Also the central steps during protocol development were related to cell viability (OD_{750} range, reduction or prohibition of electric pulse) and homologous recombination rate (longer homologous sequences), but not with DNA uptake. This is an active step during NT performed by the bacteria themselves (see 1.3.1). In contrast, no experimental data indicate a supportive effect of EP on transformation efficiency.

Crucial for the restoration of cell viability was possibly the prohibition of condensation events during cultivation of suspension culture. This changed the conditions during the 2 d regeneration time after the washing procedure and thus during the time point of NT. Also the changed OD_{750} could be critical, since it is not only an indicator of cell number but also of growth phase. For many naturally transformable bacteria it is known that natural competence is growth phase dependent (see also discussion section 4.1.1).

3.2 Kanamycin resistance, ploidy, HE10JO contamination

3.2.1 Level of kanamycin resistance

Transformants of HE10JO and HE10DO show high resistance towards the selection marker kanamycin in suspension culture. After the selection of transformants on selective agar plates they were transferred for further cultivation in selective suspension culture with elevated nitrate and phosphate concentrations ($f/2^+$ medium). The transformants were selected on f/2 agar plates with kanamycin concentration of 70 or 120 µg/ml. Long term cultivation on f/2 agar plates with 120 µg/ml kanamycin could result in propagation arrest and subsequent dying of the selected cells (see also 3.1.2). In contrast to this, transformants transferred to $f/2^+$ suspension culture tolerated kanamycin concentration up to 14 mg/ml (Figure 16). By Kevin Schulz (bachelor thesis) it was demonstrated that HE10DO transformants (7-K) can tolerate even higher kanamycin concentration. However, cell propagation decreased with increasing kanamycin concentration (tested for 20 mg/ml – 50 mg/ml). In comparison, the *Phormidium lacuna* wild type (WT) could not propagate on 50 µg/ml kanamycin.

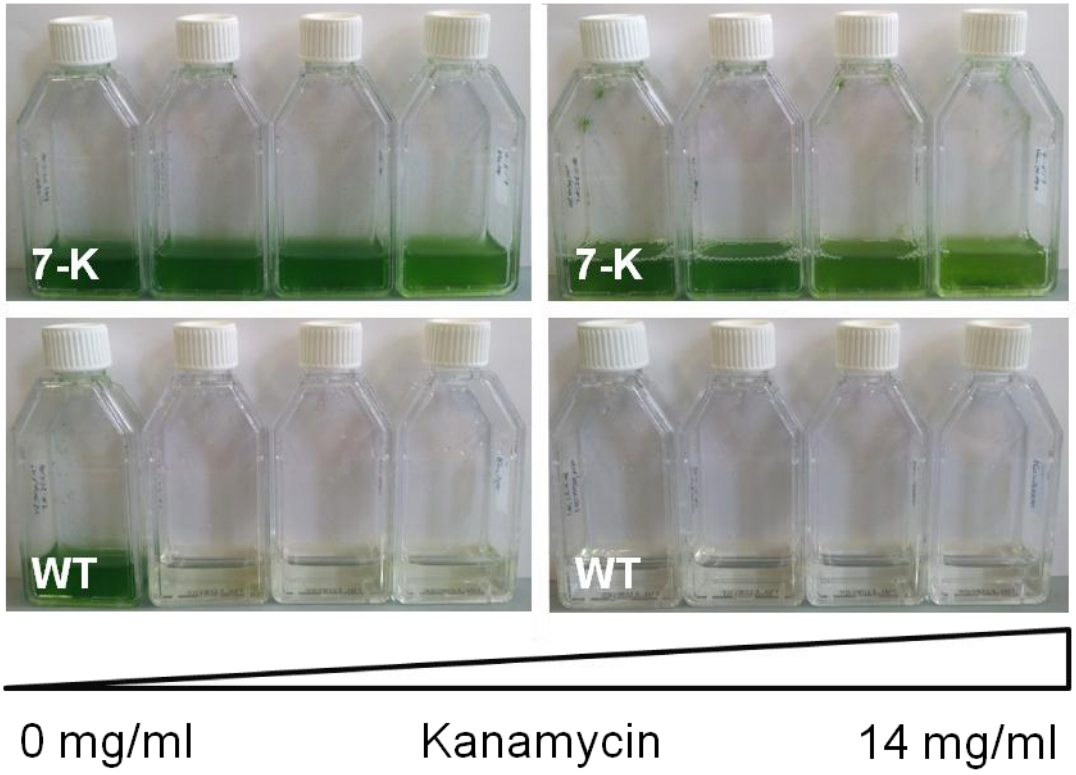


Figure 16: Resistance of HE10JO WT and 7-K towards kanamycin in a concentration dependent manner. Kanamycin concentration from left to right: 0, 50, 100, 200 µg/ml; 4.5, 8.3, 11.5, 14.3 mg/ml.

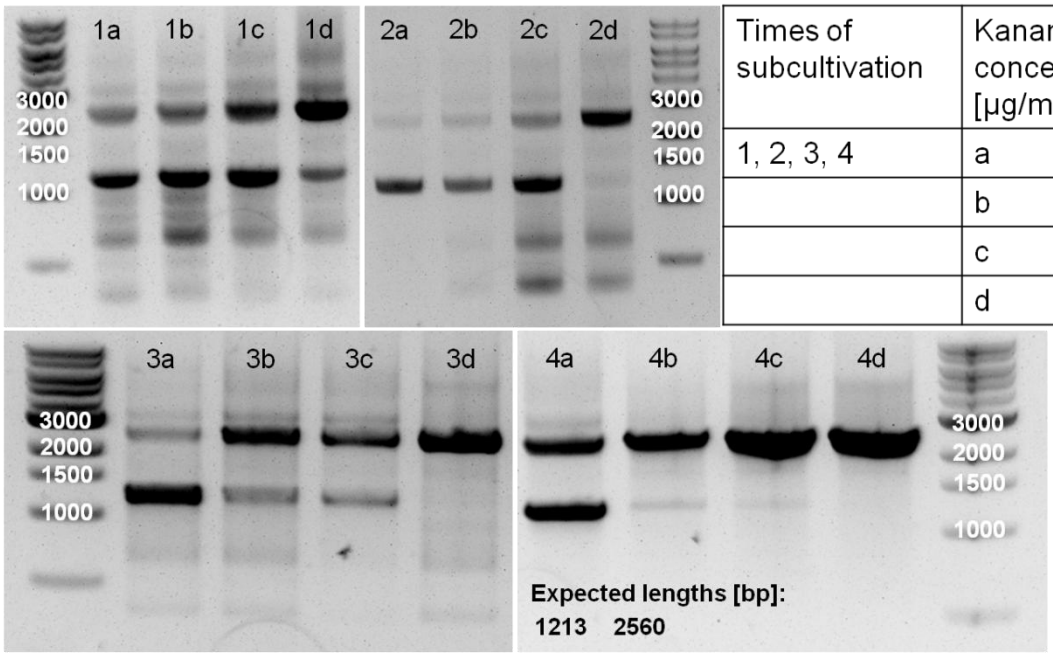
The considerably high resistance was interpreted as a possible indicator of high gene expression of the selection marker enabled through the promoter of the *kanR* operon. Experiments in this work to establish promoter assays by transforming a GFP (green fluorescent protein) encoding plasmid did not result in transformants (Table 9 and electronic supplement: transformation experiments). Thus, direct proof of promoter activity is not possible at this time.

7-P-K:2k and 7-P-A-K:2k, plasmids for ethanol production (see 3.3), have the *kanR* ORF on the second or third position after the respective promoter (Table 4 in 2.1.2). For the respective transformants of HE10DO kanamycin resistance was monitored up to concentration of 8.3 mg/ml. This maximum concentration affected 7-P-A-K:2k transformants negatively but not 7-P-K:2k transformants The 7-P-A-K:2k transformants could hardly survive this concentration, indicating that 8 mg/ml is near the upper kanamycin limit for this transformant. A possible explanation for this difference in kanamycin resistance could be that the later position of the ORF of the selection marker in the polycistronic mRNA causes lower expression rates. In this case kanamycin resistance should also be lower to a smaller extent for 7-P-K:2k in comparison to 7-K transformants. As this phenomenon was just a side observation, it was not tried to detect an upper kanamycin concentration limit for all transformants. The different ribosome binding site in comparison to 7-K variants alone is not an explanation. 7-P-A-K:2k and 7-P-K:2k have the same sequence (19 bp) upstream the start codon of the *kanR* ORF (see also electronic supplement: plasmids – maps and sequences).

3.2.2 Ploidy of Phormidium lacuna and segregation of selection marker

The validation of transformants with PCR (Figure 8) showed first indication that *Phormidium lacuna* possesses more than one chromosome copy per cell and is thus oligo- or polyploid. Oligo- and polyploidy is a common phenomenon among the cyanobacteria (see introduction for further information). The additional PCR product in Figure 8, which indicates the presence of the WT sequences in a transformed cell culture, can be explained with a not fully segregated selection marker in the genome of an oligo- or polyploid organism. Further cultivation on kanamycin favored cells with a further or fully segregated *kanR* operon in their genome and thus only the longer PCR product can be detected after longer cultivation under selective pressure. Residual WT cells, which could not propagate but still survived under antibiotic pressure, or remains of their genetic information, could be an alternative explanation for this PCR product.

To further test the hypothesis that *Phormidium lacuna* possesses more than one genome copy, a modified approach of the PCR in (Figure 17) was performed. Starting with a fresh selected suspension culture of HE10DO 7-K transformants (f/2^{+}, Kan100), four samples were subcultivated four times at four different kanamycin concentrations (0, 100, 980, 8300 µg/ml). For each sample's subcultivation, the WT and heterologous chromosome were detected by PCR. In Figure 17 the ratio of the two bands towards each other is illustrated. On the highest kanamycin concentration, the WT chromosomes could not be detected from the second subcultivation onwards, while its PCR product was still the most prominent in all other approaches. With the third and fourth subcultivation, the WT chromosomes also started vanishing for the lower kanamycin concentration. Without kanamycin, the WT product was always the prominent one, but also the heterologous chromosome is always detectable.



Times of subcultivation	Kanamycin-concentration [µg/ml]	
1, 2, 3, 4	a	0
	b	100
	c	980
	d	8,300

Figure 17: Detection of WT and recombinant chromosome in *Phormidium lacuna* HE10DO transformants. Samples were HE10DO 7-K transformants that were subcultivated one to four times on the respective kanamycin concentration after the initial cultivation in f/2^{+}, Kan100 suspension culture. Primers: F25, F28. Marker: 1 kb DNA ladder (NEB, USA).

Even though this experiment only allowed qualitative analysis, it shows that *Phormidium lacuna* has more than one chromosome per cell. An alternative explanation for the smaller PCR product would be reaming WT cells in the culture. But it is unlikely that *Phormidium lacuna* WT could survive three or four subcultivations on 980 µg/ml kanamycin, while it could not propagate at 50 µg/ml (see 3.2.1).

The degree of ploidy of *Phormidium lacuna* was further investigated by Janko Pochert (master thesis) with a self-developed assay. This assay is based on the DAPI (4′,6-Diamidin-2-phenylindol) fluorescence of DNA and the cell number. With the known genome size of *Phormidium lacuna*, the number of genome copies can be quantified. Based on the result of Janko Pochert, the polyploidy for *Phormidium lacuna* can be confirmed. He could further demonstrate that the number of chromosomes per cell decreases from early to later growth phase. This phenomenon is also described in literature for *Synechocystis* sp. PCC 6803 (Griese *et al.*, 2011, Zerulla *et al.*, 2016).

The fast segregation of the selection marker in the *Phormidium lacuna* genome and the high resistance towards kanamycin is correlated with each other. From the present results it is not possible to state if the fast segregation is a requirement of the high kanamycin resistance or if the high kanamycin resistance promotes the selection cells with fully segregated genomes (see 4.1.2). Once completely segregated, the heterologous sequence was stably integrated into the genome: Fully segregated HE10JO 7-K transformants were subcultivated several times without antibiotic pressure and maintained their kanamycin resistance in subsequent, selective cultivation. The ability for fast segregation in the genome of *Phormidium lacuna* is a beneficial feature for an organism considered for use in recombinant biotechnology, since homozygote transformants can be generated very fast after selection. Additionally, it should be highlighted that transformants of *Phormidium lacuna* can be

transferred from low kanamycin concentration (100 µg/ml) to very high kanamycin concentrations (8.3 mg/ml) without any intermediate step.

3.2.3 HE10JO and environmental contamination

In transformation experiments with the non-axenic HE10JO strain the transformation procedure strongly increased the growth of the contamination on agar plates. The orange color that remains after the dying of HE10JO samples during selection on kanamycin was a visible indication (Figure 6, WT on plates with 70 µg/ml kanamycin). This contamination is obviously less sensitive to kanamycin than *Phormidium lacuna* WT. Also other *Phormidium lacuna* samples of our workgroups collection are still non-axenic and harbor probably also this contamination. The strains GI09CO and HE10DO were also selected during this study because they were verifiably axenic.

Fabian Falk (master thesis) enabled the cultivation of this environmental contamination and identified it as belonging to the genus *Marivirga*. It is known for the sequenced species *Marivirga tractuosa* H-43T that it possesses several antibiotic resistances including aminoglycosides like kanamycin (Pagani *et al.*, 2011). This fits with the observation that Marivirga could survive on selective agar plates. It is also an explanation for the observation that in several transformation experiments with HE10JO false positive selection was reported: HE10JO sample on selective agar plates started to die, but subsequently propagation of HE10JO could be observed at first comparative with common selection. Trained observers can distinguish positive and false positive transformants in propagation and distribution behavior on the plate. Furthermore, false positive transformants could also be easily identified with the transfer from agar plates to selective suspension culture, in which false positive transformants could not propagate. False positive selection on agar plates can be explained by the *Marivirga* contamination that mediated a minor kanamycin resistance on agar plates (possibly through degradation of kanamycin), but not in suspension culture. For the axenic strain HE10DO, false positive selection was not observed. Efforts in our workgroup to remove *Marivirga* from the HE10JO stock culture did not succeed so far. A microscopy image of HE10JO and *Marivirga* is shown in Figure 18.

Figure 18: Microscopy image of *Phormidium lacuna* HE10JO and *Marivirga*. Bright field microscopy, 1000x magnification. Focus was adjusted to *Marivvirga* sp. (thin, grey and long bacteria). *Phormidium lacuna*: Green cells (ca. equal width and length) are organized in filaments.

3.3 Ethanol production

The establishment of the transformation method was an essential step to make *Phormidium lacuna* accessible for recombinant biotechnology. Subsequently, the potential of *Phormidium lacuna* for biotechnology should be examined with the recombinant production of organic compound. The compound of choice is ethanol, because it is the best characterized heterologous product in cyanobacteria.

One promoter is known to drive gene expression in *Phormidium lacuna* with the promoter of the used kanamycin operon (*kanR*). In 3.2, a possible strong promoter activity is listed as potential explanation for the high kanamycin resistance of *Phormidium lacuna* transformants. Based on the assumption that this is true, it was proceeded with the overexpression of the enzymes pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH), the relevant proteins for ethanol production, under control of the respective promoter.

3.3.1 Design of the vectors for transformation

Phormidium lacuna HE10JO possesses several enzymes with predicted alcohol dehydrogenase activity (Nies *et al.*, 2017), some with activity related to short chain alcohols. Alcohol dehydrogenases in general can have rather low substrate specificity (Nealon *et al.*, 2015). Thus, there are several enzyme candidates for catalyzing the reaction between ethanol and acetaldehyde. Also *Phormidium lacuna* HE10JO has an alternative pathway to produce ethanol starting from pyruvate with a pyruvate formate lyase (phl#3773) and a bifunctional alcohol dehydrogenase/ acetaldehyde dehydrogenase (phl#2712) (Nies *et al.*, 2017).

Based on this genetic information, the overexpression of PDC alone could be sufficient to enable heterologous ethanol production. This is also shown for *Synechocystis* sp. PCC 6803, but to achieve high production rates heterologous coexpression of ADH is necessary (Dexter *et al.*, 2015). Thus, both approaches were tried for *Phormidium lacuna*. Also it was expected that frequency of homologous recombination declines with increasing insert size and therefore obtaining transformants expressing PDC alone were expected to be easier. The sequences of *pdc* and *adh* from *Zymomonas mobilis* were used. These are commonly used sequences for ethanol production, but also the overexpression of endogenous ADH works in cyanobacteria (Dexter *et al.*, 2015).

Since there is no experience of heterologous gene expression in *Phormidium lacuna* besides the kanamycin resistance, two different designs were used for the expression of PDC or PDC and ADH together. Either they were expressed under the control of one promoter (7-P-K:2k, 7-P-A-K:2k) together with the *kanR* gene or separately with an own *kanR* promoter (7-pP_K:2k, 7-pP-A_K:2k). For schematic overview see Table 4 (in 2.1.2) and for detailed information see electronic supplement (transformation experiments and plasmid – maps and sequences). For the second group of constructs the expression on the *kanR* gene should be given, since the *kanR* operon is not changed compared to

the already characterized plasmids. A disadvantage is that two times the same promoter is present on the resulting plasmid, which could possibly enable undesired recombination events due to sequence homologies. The use of one promoter for two or three ORFs has the advantage that with resulting transformants it is very likely that not only the *kanR* gene but also the upstream encoded ORFs *pdc* and *adh* are transcribed. Since there is no experience with the heterologous transcription of di- and tricistronic mRNA in *Phormidium lacuna*, both designs were used to obtain transformants.

3.3.2 Transformants and their characterization

HE10DO transformants were obtained for the constructs 7-P-K:2k, 7-pP_K:2k, and 7-P-A-K:2k, but not for 7-pP-A_K:2k (electronic supplement: transformation experiments). The integration of the respective inserts was checked with PCR and additionally the PCR products for the full length insert were sequenced. No mutations were detected, thus the transformants were considered to be as intended. For 7-pP_K:2k, the terminator behind the ORF of *pdc* was not found in the respective transformant. This is not related to a recombination event in *Phormidium lacuna*, but it could be derived to the construction of the plasmid. Sequencing control of the plasmid revealed that possible a mixed sample with and without terminator on the respective position is existent. Since *Phormidium lacuna* transformants of 7-P-K:2k were obtained in the first transformation attempt, no effort was put into correcting the plasmid 7-pP_K:2k. Differences of the transformants could be detected for 7-PDC-ADH-K:2k in the kanamycin resistance (see 3.2.1), which was lower than for 7-K.

Notably, the plasmid 7-pP_K:2k was not constructed like intended due complications in the cloning process: The stop codon of the *pdc* ORF was deleted because the 3'UTR (untranslated region) was not integrated at this site during multiple fragment cloning. PDC's coding sequence was thereby extended by 18 amino acids by this. 7-pP_K:2k was transformed into *Phormidium lacuna* anyhow since the *kanR* operon itself was not changed and it was not obvious in the beginning if di- or tricistronic plasmid constructs would work. In potential further experiments concerning ethanol production the differing vector design and the resultant changes in the respective transformant should be considered, nevertheless. A different phenotype could not be observed between 7-pP_K:2k and 7-P-K:2k.

3.3.3 Ethanol detection

3.3.3 a) Photometry

Ethanol production of the respective transformants and HE10DO wild type (WT) as reference was initially determined in a photometric assay: With the addition of external NAD and ADH to the cell supernatant the back reaction from ethanol to acetaldehyde is catalyzed and the correlated rise in NADH concentration can be monitored with the linked increase in OD_{340}.

However, ethanol concentration in the culture supernatants was in all measurements below the detection limit of this assay. Under the in 2.4.1 described conditions, the detection limit of this assay is

between 316 mg/l (6.9 mM; weak signal) and 32 mg/l (0.7 mM; no detection) ethanol in the cell supernatant.

3.3.3 b) Ethanol detection – Gas chromatography

Since ethanol detection with an enzymatic assay was not sensitive enough, gas chromatography (GC) was used to examine the ethanol production of HE10DO transformants. Additionally, *Synechocystis* sp. PCC 6803 WT and a derived transformant supposedly capable of ethanol production (*Syn* - petJ PA) were used as controls. With GC, ethanol could be detected in the cultures supernatant. Ethanol standards were used for every experiment to define ethanol specific retention time and to generate a standard curve for ethanol concentration (Figure 22 in the supplement). The GC derived ethanol concentration in the cell supernatant was divided by the cultures OD_{750} to allow the comparison of samples with different cell densities.

The results of three independent experiments are summarized in Figure 19. It is shown that the ethanol content in relation to cultures OD_{750} is between ca. 20 – 35 µM for all samples with their standard media (BG11/TES for *Synechocystis* sp. PCC 6803 or $f/2^+$ for *Phormidium lacuna* HE10DO). This is clearly below the detection limit of the photometric assay. No difference can be monitored between the WT strains and the transformants of *Synechocystis* sp. PCC 6803 and *Phormidium lacuna* HE10DO. For HE10DO WT and the transformants 7-Kn:2k and 7-P-A-K:2k additionally the influence of additional glucose (1 mM) in the medium was tested. In this case, relative ethanol concentration was lower for WT and 7-Kn:2k in comparison to the standard medium in this case, while it was at the same level for 7-P-A-K:2k.

For all measurements used in Figure 19, ethanol peak could always be identified by the retention time. In contrast, the calculation of the signal strength (i.e. the integration of the respective peak by the software ChromStar) could not be performed properly in several cases. In most cases, the detected ethanol levels were too low to exclude interference with varying background signals. Examples of GC measurements are given in the supplement (Figure 23). These examples illustrate the difficulties in data analysis and the limitation in precise quantification. Ethanol peaks were integrated in any case but in measurements, which are comparable with Figure 23 E or F, the calculated size of the ethanol peak is rather an imprecise estimation than a precise measurement. Therefore, no t-test error probabilities are indicated in Figure 19, since they would not have any informative value. All differences between in ethanol concentration are considered insignificant due to the methods limitation.

Furthermore, the standard curve did not allow a precise conversion from peak area to ethanol concentration (problem elucidated in the supplement, see Figure 22 and the respective explanation in the text), but this only affects the conversion factor. The limitations of the available method affect the quantitative results but the method is still valuable in providing information on the relative comparison of the ethanol in the culture's supernatants.

Nevertheless, Figure 19 shows that a comparative, low ethanol level could be detected in all sample supernatants and no distinct differences exist neither between the monitored conditions nor between the different transformants and controls. Also the effects of f/2 medium (as comparison to f/2$^+$ medium) and partial incubation in darkness, respectively, were tested for *Phormidium lacuna* HE10DO (see also Table 7 in 2.4.2). It was tested if reduced levels of available nitrate and phosphate or differences in illumination (4 d under continuous illumination, 3 d in darkness in contrast to 7 d continuous illumination), respectively, have an influence on ethanol production. But most of the measurements were not evaluable due to the limitations elucidated above. However, the evaluable measurements showed that the ethanol levels were comparable to the values in Figure 19.

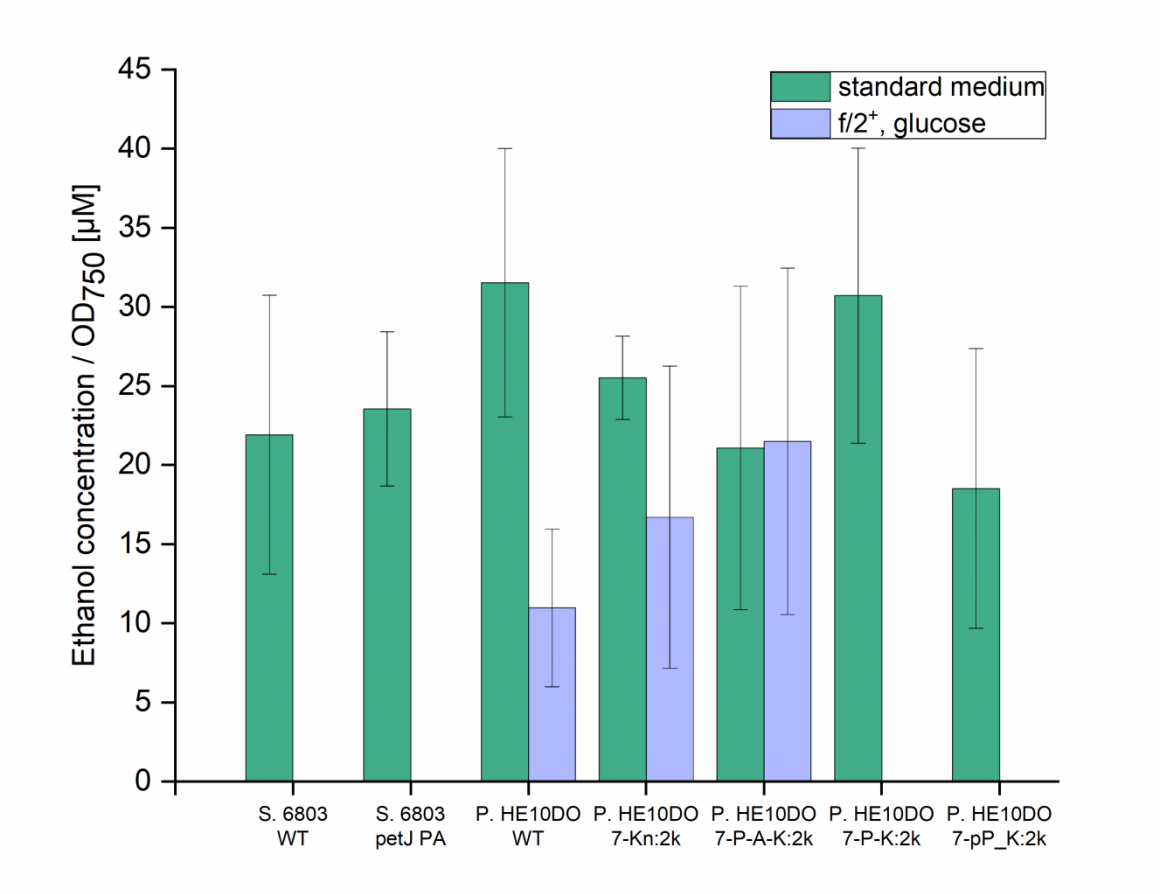


Figure 19: Ethanol concentration of culture supernatant in relation to cultures OD_{750}. Samples were WT and transformants of *Synechocystis* sp. PCC 6803 and *Phormidium lacuna* HE10DO. Standard medium is BG11 for *Synechocystis* sp. PCC 6803 and f/2$^+$ for *Phormidium lacuna* HE10DO. WT: wild type. Each bar represents the mean value of three independent experiments. Error bars show standard error. Significance: T-test error probabilities were not calculated (see text).

For the samples *Synechocystis* sp. PCC 6803 petJ PA, *Phormidium lacuna* HE10DO 7-P-A-K:2k, and possibly also for 7-P-K:2k and 7-pP_K:2k an elevated ethanol was expected. But for all this transformants potentially producing ethanol no elevated ethanol level was monitored in comparison to the controls. The measured ethanol can be explained with an endogenous ethanol production of the WT organisms. Slight differences between the different bars can be explained with the method's

limitation and statistic variations. For ethanol production, driven by heterologous gene expression, levels several orders of magnitude higher than the respective controls would be expected. Thus, no heterologous ethanol production can be demonstrated in HE10DO transformants.

Notably, the relative ethanol levels between *Synechocystis* sp. PCC 6803 and *Phormidium lacuna* HE10DO are in the same range. In fact, the absolute ethanol levels in the cultures supernatant were higher for both *Synechocystis* sp. PCC 6803 samples, but so was OD_{750}. The standardization with the OD_{750} showed that the endogenous ethanol production per cell for these quite distantly related organisms are comparable.

Even though the GC method in this form has several limitations, ethanol peaks could always be identified by the retention time for the evaluated data. This taken together with differences in the absolute concentration before standardization with the OD_{750} (see above) and the missing of an ethanol peak in a no-cell control show the specificity of this method. Thus, the finding of no heterologous ethanol production is justified irrespective of the limitations mentioned above (see also discussion section).

For a precise evaluation of the ethanol concentration in the culture's supernatant, a low background during GC measurements and a qualitative high standard curve is required. For future transformants with higher production rates also photometry based ethanol detection might be possible.

3.4 Prediction of natural competence in cyanobacteria by bioinformatics

Natural competence in cyanobacteria was often regarded as an exclusive trait of certain unicellular cyanobacteria. The finding of natural transformation (NT) in the filamentous cyanobacterium *Phormidium lacuna* raises the question if natural competence is possibly a more common trait among all or at least a bigger subgroup of cyanobacteria. NT enables efficient gene transfer into cyanobacteria like into the model organisms *Synechococcus elongatus* PCC 7942 and *Synechocystis* sp. PCC 6803 (Shestakov & Khyen, 1970, Grigorieva & Shestakov, 1982). NT also enables efficient gene transfer for *Phormidium lacuna* (see 3.1.3). Conjugation and EP are the two other common methods for gene transfers into cyanobacteria. For conjugation an additional organism like *Escherichia coli* is required while EP can have negative impacts on cell viability. The published NT protocols are uncomplicated and straight forward methods. For NT protocols in literature consider 4.2.2. Thus, NT would be the desired method for establishing a transformation protocol for a new cyanobacterium. In this section, bioinformatics was used as tool to make predictions about the distribution of natural competence in cyanobacteria. By this, it was possible to make sophisticated organism choices to test for NT in other cyanobacteria.

Proteins with proven or predicted role for NT (natural transformation factors, NTFs, see also 1.3.1) in *Synechocystis* sp. PCC 6803 were compared with the total protein derived from the sequenced genomes of 35 other cyanobacteria. Therefore, the BLASTp function of the offline NCBI tool BLAST+ (Camacho *et al.*, 2009) was used to find protein homologs to the NTFs by local alignments. The bit score in the BLASTp analysis was used as indicator for the protein with the highest homology to the respective NTF. The highest bit scores of all local alignment searches by BLASTp are listed in the electronic supplement (bioinformatics). Also the respective E values (expect values) and the alignment lengths are listed (see also 3.4.3 and 3.4.4).

The best values for the bit score in the initial BLASTp search (based on the selected NTFs of *Synechocystis* sp. PCC 6803) were used to identify the NTFs in other natural competent cyanobacteria (NCC). Based on the amino acid sequence of these NTFs the BLASTp analysis was repeated for all NCC. Thereby, for every cyanobacterium used in this analysis the protein with the highest homology (based on the bit score) towards every NTF of every NCC was identified. Based on these results it is possible to make predictions for other potential NCC.

The usage of the NTF set of all NCC has an advantage in the prediction of candidates to test for natural transformation (CNT): Not only CNT that are closely related to one cyanobacterium (like e.g. *Phormidium lacuna*) can be found based on high homology to one single NCC. Also CNT can be predicted that do not share high homology with one NCC but with all NCC in total. Thereby, more CNT could be predicted. In the analysis in this section promising candidates for natural transformation are abbreviated CNT^+, and non-promising candidates are abbreviated CNT^-.

3.4.1 Selection of cyanobacteria

There are six genera with examples for NT among the cyanobacteria. Three belong to the order Synechococcales with *Synechocystis*, *Synechococcus*, and *Thermosynechococcus* (Shestakov & Khyen, 1970, Devilly & Houghton, 1977, Stevens & Porter, 1980, Grigorieva & Shestakov, 1982, Onai *et al.*, 2004). With *Microcystis aeruginosa* PCC 7806 (Dittmann *et al.*, 1997) there is one species belonging to the order Chroococcales. These cyanobacteria are all unicellular. Natural competence was often regarded as an exclusive feature of unicellular Cyanobacteria in literature reviews. Actually, there is one single record demonstrating NT in the filamentous cyanobacterium *Nostoc muscorum* (Trehan & Sinha, 1982). But only mutant completion with exogenous DNA but no integration of foreign genes was shown in this case. In 3.1.3, it is demonstrated that NT is as well possible in *Phormidium lacuna* with inserts up to ca. 4 kb length. Thus, NT is also possible in a filamentous cyanobacterium of an order other than the Nostocales and might be more common among the cyanobacteria than generally assumed.

Since there is only a sequenced genome for the strain of HE10JO of *Phormidium lacuna*, this genomic sequence data was used for comparison. HE10DO, for which NT was demonstrated explicitly, and HE10JO have the same 16S rRNA sequence and differ in their phytochrome sequence only in one nucleotide (Nies *et al.*, 2017). Hence, their genomic data is regarded sufficient concordant. Also HE10JO is considered to be generally capable of NT and the lack of generated HE10JO transformants in NT experiments may have other reasons (see also 4.1.2).

As an initial approach to predict promising candidates to test for NT, 36 species were chosen for a broad coverage among the whole phylum with a focus on the orders with the NCC (Table 12). As NCC were chosen: *Synechocystis* sp. PCC 6803, *Synechococcus elongatus* PCC 7942, *Synechococcus* sp. PCC 7002, *Thermosynechococcus elongatus* BP-1, *Phormidium lacuna* HE10JO, and *Microcystis aeruginosa* PCC 7806. *Nostoc muscorum* is also natural competent but it does not have a sequenced genome. Therefore, it could not be added to the list of NCC. Nevertheless, two other *Nostoc* species and three other representatives of the Nostocales were selected in order to have also a focus on this order.

Table 12: Cyanobacteria used in comparative bioinformatics analysis of NTFs. The bold characters indicate the strains capable of NT as well as the corresponding families and orders. For distinct identification NCBI taxonomy ID and RefSeq are listed. Taxonomic classification according the NCBI database.

Order	Family	Species/strain	NCBI taxonomy ID RefSeq
Gloeobacterales	Gloeobacteraceae	*Gloeobacter violaceus* PCC 7421	txid251221 GCF_000011385.1
Synechococcales	Leptolyngbyaceae	*Leptolyngbya boryana* PCC 6306	txid272134 GCF_000353285.1
	Merismopediaceae	***Synechocystis* sp. PCC 6803**	txid1148 GCF_000270265.1
		Synechocystis sp. PCC 7509	txid927677 GCF_000332075.2
	Prochloraceae	*Prochlorococcus marinus* subsp. marinus str. CCMP1375	txid167539 GCF_000007925.1
	Pseudanabaenaceae	*Pseudanabaena* sp. PCC 7367	txid82654 GCF_000317065.1

Table 12 (continued): Cyanobacteria used in comparative bioinformatics analysis of NTFs. The bold characters indicate the strains capable of NT as well as the corresponding families and orders. For distinct identification NCBI taxonomy ID and RefSeq are listed. Taxonomic classification according the NCBI database.

	Synechococcaceae	*Cyanobium gracile* PCC 6307	txid292564 GCF_000316515.1
		***Synechococcus elongatus* PCC 7942**	txid1140 GCF_000012525.1
		***Synechococcus* sp. PCC 7002**	txid32049 GCF_000019485.1
		Synechococcus sp. PCC 7502	txid1173263 GCF_000317085.1
		***Thermosynechococcus elongatus* BP-1**	txid197221 GCF_000011345.1
Oscillatoriales	Coleofasciculaceae	*Geitlerinema* sp. PCC 9228	txid111611 GCF_001870905.1
	Cyanothecaceae	*Cyanothece* sp. ATCC 51142	txid43989 GCF_000017845.1
	Microcoleaceae	*Arthrospira platensis* NIES-39	txid696747 GCF_000210375.1
		Trichodesmium erythraeum IMS101	txid203124 GCF_000014265.1
	Oscillatoriaceae	*Oscillatoria acuminata* PCC 6304	txid56110 GCF_000317105.1
		Oscillatoria nigro-viridis PCC 7112	txid179408 GCF_000317475.1
		Phormidium ambiguum IAM M-71	txid454136 GCF_001904725.1
		***Phormidium lacuna* HE10JO**	txid1765956 GCF_900149785.
		Phormidium sp. OSCR	txid1666905 GCA_001314905.1
		Phormidium tenue NIES-30	txid549789 GCF_001904775.1
		Phormidium willei BDU 130791	txid399101 GCF_001637315.1
Chroococcales	Aphanothecaceae	*Halothece* sp. PCC 7418	txid65093 GCA_000317635.1
	Chroococcaceae	*Geminocystis herdmanii* PCC 6308	txid113355 GCF_000332235.1
	Microcystaceae	*Microcystis aeruginosa* NIES-843	txid449447 GCF_000010625.1
		***Microcystis aeruginosa* PCC 7806**	txid267872 -
		Microcystis aeruginosa PCC 7806SL	txid1903187 GCF_002095975.1
Pleurocapsales	Dermocarpellaceae	*Stanieria cyanosphaera* PCC 7437	txid111780 GCF_000317575.1
Spirulinales	Spirulinaceae	*Spirulina major* PCC 6313	txid129961 GCF_001890765.1
Chroococcidiopsidales	Chroococcidiopsidaceae	*Chroococcidiopsis thermalis* PCC 7203	txid251229 GCF_000317125.1
Nostocales	Hapalosiphonaceae	*Fischerella muscicola* PCC 7414	txid306281 GCF_000317205.1
	Nostocaceae	*Anabaena cylindrica* PCC 7122	txid272123 GCF_000317695.1
		Anabaena sp. 90	txid46234 GCF_000312705.1
		Nostoc punctiforme PCC 73102	txid63737 GCF_000020025.1
		Nostoc sp. PCC 7120	txid103690 GCF_000009705.1
	Rivulariaceae	*Calothrix* sp. PCC 6303	txid1170562 GCF_000317435.1

3.4.2 Selection of natural transformation factors

Synechocystis sp. PCC 6803 is the only cyanobacterium, for which genes involved in natural competence were characterized experimentally (Bhaya *et al.*, 1999, Bhaya *et al.*, 2000, Yoshihara *et*

al., 2001, Okamoto & Ohmori, 2002). Furthermore, NT has been characterized more extensively in other bacteria (see also introduction, especially for mechanism of NT).

Fourteen proteins were considered to be essential for NT in *Synechocystis* sp. PCC 6803 (Figure 4, Table 13). The type IV pilus enables the DNA transport across the outer membrane. Sigma factor F (SigF) controls the expression of *pil* genes in *Synechocystis* sp. PCC 6803 and was therefore added to the selection (Table 13). Proteins of the ComEA-, ComEC-, and ComF-family (all involved in DNA transport across the inner membrane) were considered irrespective of experimental proof or not. Same is true for DprA (protection of single stranded DNA in the cytoplasm), and RecA (homologous recombination).

Table 13: List of NTFs in *Synechocystis* sp. PCC 6803. These proteins were proven or considered to be essential for NT due to their annotation. Protein name for the competence factors (Com proteins) is orientated on the domain responsible for the annotation (annotation in the NCBI database partially contradictive). Function of the protein in respect to NT was either derived from the literature for *Synechocystis sp.* PCC 6803 mutants (indicated on the right) or complemented with information of the gram negative bacterium *Vibrio cholerae* (Matthey & Blokesch, 2016).

Protein	Gene ID	Function	Literature
ComEA-family	*slr0197*	DNA binding in periplasm	(Yoshihara *et al.*, 2001)
ComEC-family	*sll1929*	inner membrane channel	-
ComF-family	*slr0388*	linked with DNA transport into cytoplasm	(Nakasugi *et al.*, 2006)
DprA	*slr1197*	protection of single stranded DNA recruitment of RecA	-
PilA1	*sll1694*	major pilus subunit	(Yoshihara *et al.*, 2001)
PilB1	*slr0063*	pilus polymerization	(Yoshihara *et al.*, 2001)
PilD	*slr1120*	prepilin peptidase	(Bhaya *et al.*, 2000)
PilM	*slr1274*	unknown, part of type IV pilus	(Yoshihara *et al.*, 2001)
PilN	*slr1275*	unknown, part of type IV pilus	(Yoshihara *et al.*, 2001)
PilO	*slr1276*	unknown, part of type IV pilus	(Yoshihara *et al.*, 2001)
PilQ	*slr1277*	outer membrane pore	(Yoshihara *et al.*, 2001)
PilT	*slr0161*	pilus depolymerization	(Okamoto & Ohmori, 2002)
RecA	*sll0569*	homologous recombination	-
SigF	*slr1564*	Sigma factor	(Bhaya *et al.*, 1999)

3.4.3 BLASTp with NTF query

Fourteen NTFs of *Synechocystis* sp. PCC 6803 was used as query for a BLASTp search against the total protein sequences of 36 cyanobacteria (including *Synechocystis* sp. PCC 6803). For the other NCC, one distinct result with the highest homology (highest bit score) towards *Synechocystis* sp. PCC 6803 was found for all NTFs. One exception is the protein PilA1, the mayor subunit of the type IV pilus. It is reported for *Synechocystis* sp. PCC 6803 that its genome encodes different subtypes of PilA (Yoshihara *et al.*, 2001). In the other NCC, two or more proteins had often similar bit scores for the alignment with PilA1 of *Synechocystis* sp. PCC 6803. In this case always the alignment with the

highest bit score was considered. As there were at least one hit for PilA1 and all other type IV pilus related NTFs were present, the pilus of all NCC was considered functional.

Thereby, the NTFs of the other NCC were identified based on their homology towards the NTFs of *Synechocystis* sp. PCC 6803. The NTFs of the other NCC were used as a query for BLASTp with the selected cyanobacteria in Table 12. The results for the highest values of the bit score were listed together with the respective E value and alignment length for every BLASTp search in the electronic supplement (bioinformatics/raw data). The annotation of the identified proteins differed in some cases from the function of the NTFs. A differing annotation for the pili proteins is not contradictive since they can also be included in other functions (Berry & Pelicic, 2015). In some other cases the proteins with the highest homology towards the NTF did not have annotated function. Therefore, the annotations of the homolog proteins were not considered.

3.4.4 Interpretation of the BLASTp results

The NCC are known to be capable of NT. Therefore, the BLASTp outcomes for the NCC were compared with the results of the other cyanobacteria to predict CNT^+ (see 3.4.5 and 3.4.6). The considered parameters were bit score, E value and alignment length. Bit score is the most important outcome of a BLAST search. Bit score shows the quality of an alignment and is normalized, so different searches can be compared. A high bit score indicates high homology between two sequences. The E value and the alignment length were considered as controls for the bit score. The E value indicates the probability to find an alignment with the same bit score in the database by chance. A low E value indicates a high significance of the results. Alignment length indicates if bit score of an alignment corresponds to only a highly conserved sequence section or a lower homology but over the full length sequence.

3.4.5 Minimum homology quotient

One approach to make a prediction for new CNT based on homology search was the following: Those cyanobacteria were considered promising that share higher homology with one NCC than this NCC share with the least related other NCC (minimum homology). Therefore, the BLASTp outcomes, which indicated the lowest homology among the NCC, were listed for all NTFs (lowest bit score, highest E value, shortest alignment length). These were the minimum homology values. The values for bit score, E value, and alignment length of a BLASTp search were divided by the respective minimum homology values. This resulted into the respective minimum homology quotient (see formula below). This calculation was performed for every BLASTp search in this work (electronic supplement: bioinformatics/minimum homology quotient). If the minimum homology quotient is above 1, then for this cyanobacterium's NTF the respective value is above minimum homology. If this was true for the bit score of all 14 NTFs, a cyanobacterium was classified as potential naturally transformable with this

approach (CNT^+) if not it was classified as CNT^-. The minimum homology quotient was calculated for the E values and the alignment lengths as controls.

Bit score and alignment length are linear values while the E value is an exponential value. To have a way to treat all value the same, only the exponent of the E values were considered to calculate the minimum homology value and also later the mean value (3.4.6). Very small E values were rounded to zero automatically by the BLASTp program. This interferes with the analysis. Therefore, corresponding E values were set to 10^{-200}.

The minimum homology quotients are listed in the electronic supplement (bioinformatics/minimum homology quotient) and they are indicated in a color-code. A three color code was used for the quotient for bit score and exponent of the E value: The level of minimum homology, 1, was labeled with yellow. A comparative higher homology, 1.5 and higher, was labeled green while quotients below minimum homology, 0.5 and lower, were red. Values between 0.5 and 1.5 were labeled in mixtures of these colors (see color code below). The alignment length was a control to monitor if full length sequence was compared or just a subsection of the protein. Therefore a two color code was used. A quotient of 1 was labeled blue and indicated full length comparison while a quotient of 0.5 was red. The quotients between these two values were labeled as mixtures of blue and red.

$$\frac{BLASTp\ outcome}{minimum\ homology\ value} = minimum\ homology\ quotient$$

0.5 1 1.5

For the alignment of comEA of *Synechocystis* sp. PCC 6803 with the total protein sequences of *Gloeobacter violaceus* PCC 7421 the minimum homology quotient for the bit score is calculated as an example. The protein of *Gloeobacter violaceus* PCC 7421 with the highest homology towards comEA of *Synechocystis* sp. PCC 6803 had a bit score of 351 in the BLASTp analysis (see also electronic supplement: bioinformatics/raw data). The NCC, which shared the lowest homology with *Synechocystis* sp. PCC 6803 regarding comEA amino acid sequence, was *Synechococcus elongatus* PCC 7942. The respective bit score was with 395 bigger than the respective bit score of *Gloeobacter violaceus* PCC 7421. Thus, the resulting minimum homology quotient was with 0.89 ($\frac{351}{395}$) lower than 1. This means the level of minimum homology was not reached in this case. The respective color-code in the table in the electronic supplement (bioinformatics/minimum homology quotient: first colored field on the upper left side) is bright orange. The color-code for the minimum homology quotient allowed an overview in NTF homology of all cyanobacteria towards all NCC regarding bit score and the two control parameters E value and alignment length.

An additional example for the color-code in the electronic supplement (bioinformatics/minimum homology quotient) is listed in Table 14. Here, the minimum homology quotients for the bit score of all alignments with the total protein of *Phormidium willei* BDU 130791 are listed. Based on the color-code above it is shown that the minimum homology level of 1 was reached for nearly all alignments with the NTFs. The only exception was the alignment with the PilA1 homolog NTF of *Synechococcus elongatus* PCC 7942. The highest homology concerning the NTFs shares *Phormidium willei* BDU 130791 with *Phormidium lacuna* HE10JO. The species *Phormidium willei* was already identified as close relative of *Phormidium lacuna* in core-proteome based phylogenetic analysis (Nies *et al.*, 2017). *Phormidium willei* BDU 130791 was classified as CNT^{+} based on the analysis in Table 14 (see also Table 15). The color-codes for bit score, E value, and alignment length of all alignments are listed in the electronic supplement (bioinformatics/minimum homology quotient).

Table 14: Minimum homology quotients of the alignment of the total protein of *Phormidium willei* BDU 130791 with the NTFs of all NCC. The minimum homology level of 1 was reached for most of the alignments. The highest homology shares *Phormidium willei* BDU 130791 with *Phormidium lacuna* HE10JO. The highest value of the minimum homology quotients (hMHQ) for all NTFs is listed in bold characters. S. 6803: *Synechocystis* sp. PCC 6803, P. HE10JO: *Phormidium lacuna* HE10JO, S. 7942: *Synechococcus elongatus* PCC 7942, S. 7002: *Synechococcus* sp. PCC 7002, T. BP-1: *Thermosynechococcus elongatus* BP-1, M. 7806: *Microcystis aeruginosa* PCC 7806.

NTFs	S. 6803	P. HE10JO	S. 7942	S. 7002	T. BP-1	M. 7806	hMHQ
ComEA	1.20	2.40	1.12	1.25	1.22	1.29	**2.40**
ComEC	1.18	5.64	1.26	1.44	1.35	1.15	**5.64**
ComF	1.45	3.74	1.20	1.76	1.64	1.41	**3.74**
DprA	1.21	2.46	1.15	1.23	1.10	1.21	**2.46**
PilA1	1.05	3.92	0.95	1.05	1.13	1.11	**3.92**
PilB1	1.03	1.65	1.03	1.04	1.04	1.00	**1.65**
PilD	1.17	2.52	1.09	1.18	1.15	1.22	**2.52**
PilM	1.31	2.59	1.07	1.36	1.32	1.34	**2.59**
PilN	1.28	6.09	1.54	1.59	2.21	1.31	**6.09**
PilO	1.00	8.04	1.22	1.51	1.11	1.07	**8.04**
PilQ	2.39	7.50	1.02	1.28	1.50	2.09	**7.50**
PilT	1.00	1.52	1.00	1.00	1.00	1.00	**1.52**
RecA	1.01	1.38	1.05	1.06	1.03	1.04	**1.38**
SigF	1.08	1.90	1.05	1.08	1.16	1.11	**1.90**

In the comparison of the minimum homology quotients it is demonstrated that the homology of the NCC among each other is not considerably high (electronic supplement: bioinformatics/minimum homology quotient). Also the homologies in the order Synechococcales, even inside the species *Synechococcus* are not substantially different than to many other cyanobacteria. The highest homology

among the NCC concerning the NTFs could be found between *Synechocystis* sp. PCC 6803 and *Microcystis aeruginosa* PCC 7806.

To be classified as CNT^+ a cyanobacterium only has to share a minimum homology quotient of 1 or higher for all NTF with one NCC. Therefore, also the highest minimum homology quotient was listed (see Table 14 and electronic supplement: bioinformatics/minimum homology quotient). If only the best (i.e. the highest) minimum homology quotients were considered, for the NCC the result was of course always the self alignment. The self alignments of the NCC were helpful to see, what the max possible values for the quotients are. Also it shows that three proteins are highly conserved: PilB, PilT, and RecA. For these proteins the E value was always very low for all NCC and thus the respective quotient is always near 1. Also the highest minimum homology quotient for the bit scores was lower for RecA than for the other NTFs. In fact, RecA is highly conserved among the monitored cyanobacteria. It is the only protein that can be detected in *Prochlorococcus*, which shows lowest homology to all other NTFs. This is in accordance with literature since RecA is a highly conserved protein among the bacteria (Eisen, 1995). PilB and PilT are the ATPases that drive the type IV pilus elongation and retraction. The high conservation among many cyanobacteria is possibly necessary to maintain the enzymatic function.

For an undiscovered, natural competent cyanobacterium it would be sufficient to have a high minimum homology quotient for every NTF with at least one single NCC. Because the homology of NTF among the NCC is rather low, many cyanobacteria reach minimum homology quotient of 1 or higher if only the respective highest minimum homology quotient is considered. The minimum homology quotients of the bit scores was considered most relevant to decide if a cyanobacterium is a classified as CNT^+ or CNT^-. E value and alignment length were controls to monitor bit scores quality.

In Table 15 cyanobacteria are classified into three groups: All highest minimum homology quotients (bit score) above 1; one single quotient only slightly below 1; at least one quotient explicit below 1. Most cyanobacteria were sorted into the first group and are thus considered to be CNT^+ regarding this prediction method. The representation of the orders in this group is interesting. For the four orders with the naturally transformable cyanobacteria only Oscillatoriales, Chroococcales and Nostocales have representatives listed here. For the Synechococcales most members are classified as CNT^-, except one. This *Synechocystis* species is listed in the middle column because here only one quotient (for PilA) is slightly below threshold. Thus, this organism may still be considered as CNT^+. This is in contrast to the fact that most cyanobacteria known for NT (four of the six NCC) belong to the order Synechococcales. The orders Oscillatoriales and Nostocales only have one member each classified as CNT^- in Table 15.

Table 15: Cyanobacteria grouped after highest minimum homology quotient of the bit score. Either all quotients are above 1 for all NTFs, one quotient is between 0.95 and 1 and all others quotients are above 1, or at least one quotient is below 0.95. Number behind strain name refers to the sum of the highest minimum homology quotient of the bit score. NCC are listed below as reference of the maximum values possible for sum of quotients.

CNT$^+$				CNT$^-$	
all quotients ≥ 1		0.95 ≤ one quotient < 1; all other quotients ≥ 1		at least one quotient < 0.95	
Leptolyngbya boryana PCC 6306	20.8	*Synechocystis* sp. PCC 7509	20.8	*Gloeobacter violaceus* PCC 7421	13.8
Geitlerinema sp. PCC 9228	21.6	*Trichodesmium erythraeum* IMS101	20.4	*Prochlorococcus marinus* subsp. marinus str. CCMP1375	4.2
Cyanothece sp. ATCC 51142	25.5	*Nostoc punctiforme* PCC 73102	21.2	*Pseudanabaena* sp. PCC 7367	17.8
Arthrospira platensis NIES-39	21.5			*Cyanobium gracile* PCC 6307	9.8
Oscillatoria acuminata PCC 6304	21.4			*Synechococcus* sp. PCC 7502	17.8
Oscillatoria nigro-viridis PCC 7112	21			*Phormidium ambiguum* IAM M-71	20.1
Phormidium sp. OSCR	46.1			*Halothece* sp. PCC 7418	19.4
Phormidium tenue NIES-30	19.5			*Microcystis aeruginosa* PCC 7806SL	44.8
Phormidium willei BDU 130791	51.3			*Chroococcidiopsis thermalis* PCC 7203	21.4
Geminocystis herdmanii PCC 6308	21.5			*Fischerella muscicola* PCC 7414	21.3
Microcystis aeruginosa NIES-843	38.7				
Stanieria cyanosphaera PCC 7437	23.4				
Spirulina major PCC 6313	19.1				
Anabaena cylindrica PCC 7122	22				
Anabaena sp. 90	21.4				
Nostoc sp. PCC 7120	22.5				
Calothrix sp. PCC 6303	21.6				

NCC	
Synechocystis sp. PCC 6803	53.7
Synechococcus elongatus PCC 7942	55.8
Synechococcus sp. PCC 7002	58.7
Thermosynechococcus elongatus BP-1	54.8
Phormidium lacuna HE10JO	52.5
Microcystis aeruginosa PCC 7806	47.2

For orders with only one representative in this study, only Pleurocapsales and Spirulinales were listed as CNT$^+$, while the members of the Gloeobacterales and Chroococcidiopsidales were classified as CNT$^-$. Since also the orders with more representatives have one or more members classified as CNT$^-$ it may be by chance that a member with or without potential for NT was chosen for this analysis. A broader spectrum of cyanobacteria monitored can overcome this limitation. However, the results of this analysis can be seen as a first indication, which orders are more likely to contain CNT$^+$.

Cyanobacteria classified as non-promising candidates could be divided into two subgroups. *Gloeobacter* (Gloeobacterales) as well as *Prochlorococcus* and *Cyanobium* (Synechococcales) have an overall low homology towards the NTFs of the NCC. The other, bigger group misses only sufficient homology in one or two NTFs (see electronic supplement: bioinformatics/minimum homology quotient). The best example for this is the comparison of the three *Microcystis aeruginosa* strains in

the selection of this analysis. The NCC strain PCC 7806 shares high homology with the strain NIES-843 that is also classified as CNT^+. But the substrain SL of *Microcystis aeruginosa* PCC 7806 misses the ComEA-family protein and is thus classified as CNT^-.

This could mean that the order Gloeobacterales as well as subgroups of the Synechococcales are not capable of NT in general. In the other orders one single missing NTF may be caused by evolutionary loss. Also poor quality in the sequencing results may explain the missing of one gene in the used database.

For the classification as CNT^+, it is at first important that all quotients are equal or above 1. To further characterize the classified cyanobacteria the sum of all highest minimum homology quotients was calculated. The organisms with a higher sum share higher homology with NTFs of certain NCC and are thus more likely to perform NT. In Table 15 these numbers are listed. For *Phormidium lacuna* HE10JO close relatives are listed with *Phormidium* sp. OSCR and *Phormidium willei* BDU 130791 (see also Table 14) indicated by the high sum of quotients. Same is true for and *Microcystis aeruginosa* PCC 7806 (mentioned above). This close relationship between these species is also demonstrated by the color-code in the electronic supplement (bioinformatics/minimum homology quotient). The sum of the highest minimum homology quotient for all other promising candidates is between 19 and 26. The cyanobacteria with an overall low homology towards the NTFs of the NCC (mentioned above) also have the smallest sum of the highest minimum homology quotient.

It is quite obvious that close relatives of NCC can be considered as CNT^+. The sum of the highest minimum homology quotient supports this assumption. Therefore, these are the most promising candidates to test for NT. Especially for *Phormidium lacuna* two other closely related *Phormidium* species could be identified to test the hypothesis that NT is a common trait among this genus.

For other cyanobacteria, sum of quotients in Table 15 are too close to each other to make a sharp ranking. *Cyanothece* sp. ATCC 51142 is an exception with a value of 25.5 and 2.1 difference to the next organism. But in general all organisms in the left column of Table 15 are equally promising candidates to test for natural transformation (besides the *Phormidium* and *Microcystis* strains mentioned above).

3.4.6 Homology deviation

A limitation in the comparison through the sum of the highest minimum homology quotients was that every NTF was treated equally. The differences in the conservation of the respective NTFs among the cyanobacteria were not included. In this approach, the degree of conservation was implemented by including the standard deviation of the mean value of the BLASTp outcomes (bit score, exponent of the E value, alignment length) of all NTF. Therefore, the mean value of the BLASTp outcomes was subtracted from each respective BLASTp outcome and the difference was divided by the respective mean value's standard deviation (see formula below). The resulting value is denominated homology deviation. This was performed for every BLASTp outcome of this study. Again a three color code (red

– yellow – green) was used but with the limits -2; 0; and 2 to visualize differences in electronic supplement (bioinformatics/homology deviation). For alignment length again the two color code with red (-0.5) and blue (0) was used.

$$\frac{BLASTp\ outcome - mean\ value}{\sigma} = homology\ deviation$$

-2 0 2

Again the alignment of the NTF comEA of *Synechocystis* sp. PCC 6803 with the total protein sequences of *Gloeobacter violaceus* PCC 7421 is used as example for the calculation. As mentioned above the bit score for this alignment was 351. The mean value of the bit score for all alignments with comEA of *Synechocystis* sp. PCC 6803 was 427 and the respective standard deviation was 138. Thus, the respective homology deviation was - 0.6 ($\frac{351-427}{138}$). This means that the difference between this bit score and the mean value of the bit score for all alignments with comEA of *Synechocystis* sp. PCC 6803 was 0.6 times the standard deviation of the bit score. A negative homology deviation indicates that the bit score is smaller than the respective mean value. A positive homology deviation indicates that the bit score is bigger than the respective mean value. The color-code for this example in the table in the electronic supplement (bioinformatics/homology deviation: first colored field on the upper left side) is orange. The homology deviation was also calculated for the two control parameters E value and alignment length. For the E value only the exponent was considered (see also 3.4.5).

The primary data stayed the same between this type of processing (homology deviation) and the previous one (minimum homology quotient). Thus, no big differences were expectable, but the new kind of analysis allowed a different perspective. With the inclusion of mean value and standard deviation it was possible to state if the homology of an NTF of a cyanobacterium was in the range of one or two times of the standard deviation above or below the average homology towards an NTF of a NCC.

This analysis showed that the homologies between certain members of the NCC are below the mean overall homology. Especially *Synechococcus elongatus* PCC 7942 and *Phormidium lacuna* HE10JO share low homology with each other and the remaining NCC (electronic supplement: bioinformatics/homology deviation). The partially low homology between the NCC had implications for the prediction of CNT^+ based on homology. Since the homology between the NCC is quite low, it was not possible for the examined cyanobacteria to reach high homology levels with all NCC. Rather two patterns of homology distribution are indicators for a CNT^+: First – high to very high homology with a naturally competent cyanobacterium (relevant for close related strains or species, examples for *Phormidiu*m and *Microcystis* are mentioned in 3.4.5 and are also monitored by the homology deviation). Second – medium to high homology with several or all NCC. This second case is more

interesting, because by this way also promising candidates can be predicted that are not closely related to any NCC. The color code is a helpful tool to find promising candidates per eye when screening the whole data set (electronic supplement: bioinformatics/homology deviation). *Cyanothece* sp. ATCC 51142, *Stanieria cyanosphaera* PCC 7437 and members of the Nostocales show a widespread and higher homology towards the NCC.

Even though the homology deviation of a cyanobacterium towards every NTF of all NCC is interesting and should be considered as well, a more compact analysis is helpful. Therefore, from the homology deviation (of the bit score of every BLASTp outcome) for the six different NCC only the highest value for homology deviation was considered or the average of the homology deviation was calculated (electronic supplement: bioinformatics/homology deviation). These values were named highest homology deviation and average homology deviation, respectively. In Table 16 the cyanobacteria of this study are grouped by the smallest value of highest homology deviation. Furthermore, the mean of the average homology deviation was calculated (right column to every cyanobacterium in Table 16).

First, cyanobacteria were classified considering the smallest values of the highest homology deviation. Thus, it was monitored if all NTFs are present in a cyanobacterium or not. Considering the low homology among the NCC, the presence of an NTF could be only negated if the smallest value is below -1. Thus, the respective organism was excluded from the CNT^+. While the range of the smallest values of the highest homology deviation is quite narrow (0 to -0.6) between cyanobacteria without a close relative among the NCC, there is a gap to the CNT^- (< 1). Thus, there was a sharp tool to differentiate between cyanobacteria that miss at least one NTF and the ones that were predicted to possess all of them. In order to have a single value on how good the overall homology to all NCC is, the mean of the average homology deviation was calculated. It shows that *Cyanothece* sp. ATCC 51142 has the best overall homology, since the higher values for the organisms in the left column (smallest value of the highest homology deviation is > 1) relates to the close relation to one single NCC (as also shown above; see also electronic supplement: bioinformatics/homology deviation). How promising candidates with a mean of the average homology deviation above 0 might be, could be derived if the self-alignment was excluded for the NCC. For model organism *Synechococcus elongatus* PCC 7942 this value is even lower than for every CNT^+. This underlines the classification of every single cyanobacterium identified as a CNT^+ and underlines the preference on those cyanobacteria with a higher mean of the average homology deviation.

Table 16: Cyanobacteria grouped by smallest value for the highest homology deviation (bit score of every BLASTp outcome). Organisms were grouped in: Close relatives of certain NCCs (> 1), cyanobacteria with every NTF detected (> 0; > -0.3; > -0.6) and cyanobacteria with at least one NTF undetected (< -1). The values on the right to the organisms name are the mean of the average homology deviation (a). NCC are listed below as reference. The average mean values of all NTFs are listed with (a) and without (b) self alignment for the NCC.

CNT^+								CNT^-	
smallest value of the highest homology deviation is									
> 1		> 0		> -0.3		> -0.6		< -1	
organism	(a)	organism	(a)	organism	(a)	organism	(a)	organism	(a)
Phormidium sp. OSCR	0.5	*Cyanothece* sp. ATCC 51142	0.5	*Leptolyngbya boryana* PCC 6306	0.2	*Synechocystis* sp. PCC 7509	0.1	*Gloeobacter violaceus* PCC 7421	-0.9
Phormidium willei BDU 130791	0.5	*Arthrospira platensis* NIES-39	0.1	*Geitlerinema* sp. PCC 9228	0.1	*Pseudanabaena* sp. PCC 7367	-0.2	*Prochlorococcus marinus* subsp. marinus str. CCMP1375	-2.2
Microcystis aeruginosa NIES-843	0.7	*Stanieria cyanosphaera* PCC 7437	0.3	*Trichodesmium erythraeum* IMS101	0	*Phormidium ambiguum* IAM M-71	0.1	*Cyanobium gracile* PCC 6307	-1.4
		Nostoc sp. PCC 7120	0.3	*Oscillatoria acuminata* PCC 6304	0.1	*Geminocystis herdmanii* PCC 6308	0.2	*Synechococcus* sp. PCC 7502	-0.4
				Oscillatoria nigro-viridis PCC 7112	0.1	*Chroococcidiopsis thermalis* PCC 7203	0.1	*Halothece* sp. PCC 7418	0
				Phormidium tenue NIES-30	0	*Nostoc punctiforme* PCC 73102	0.1	*Microcystis aeruginosa* PCC 7806SL	0.5
				Spirulina major PCC 6313	0	*Calothrix* sp. PCC 6303	0.2	*Fischerella muscicola* PCC 7414	0.1
				Anabaena cylindrica PCC 7122	0.2				
				Anabaena sp. 90	0.2				

NCC		
organism	(a)	(b)
Synechocystis sp. PCC 6803	1.3	0.1
Synechococcus elongatus PCC 7942	0.5	-0.3
Synechococcus sp. PCC 7002	0.7	0
Thermosynechococcus elongatus BP-1	0.6	-0.1
Phormidium lacuna HE10JO	0.5	0.1
Microcystis aeruginosa PCC 7806	0.7	0.3

3.4.7 Conclusion

To make a prediction of CNT^+, it was searched for homologs of the NTFs of NCC in a representative selection of cyanobacteria with BLASTp. The bit score of a local alignment (BLASTp) was used as indicator of homology. Two methods were used to process the data output of the alignments: In the first method, minimum homology quotient, the output for every cyanobacterium was directly compared with the NCC. In the second approach the output of all cyanobacteria (including the NCC) was set into relation of the mean outcome of the BLASTp search (homology deviation).

The minimum homology quotient has the advantage that every cyanobacterium is set into direct relation to the NTF of the NCC. With the resulting classification it is ensured that all CNT^+ have at least with one NCC a higher homology than another NCC for every single NTF. The prediction of CNT^+ relied only on the NCC that are already known to be naturally competent.

The homology deviation method uses the overall mean and its standard deviation for comparison. It thus introduces a statistical value and makes the homologies of the different organisms better

comparable with each other. While for calculation of the minimum homology deviation of a BLASTp outcome only the NCC are relevant, for the calculation of the homology deviation all cyanobacteria used in the analysis are important. Thus, the homology deviation can only be used if the there are differences in the homology towards the NTFs of the NCC among the selected cyanobacteria. For example, if all selected cyanobacteria would miss the same NTF, it would be hard to monitor this by the homology deviation. The use of another method to process the raw data like the calculation of the minimum homology deviation is thus required as comparison to the homology deviation. Also the use of six NCC instead of less or even one single NCC is helpful. The homology deviation illustrates the low homology between the different NCC more explicitly than the minimum homology deviation. It helps to define a threshold to distinguish between promising and non-promising candidates. As a result in comparison to the first method (Table 15), in the second method (Table 16) less cyanobacteria are classified as non-promising. This can also be justified with the NCC's low homology deviation (without self-alignment).

Both ways predict with small differences a broad spectrum of cyanobacteria that are, based on an NTF homology approach, promising candidates to test for NT. Both analyses as well as the single results for every single naturally competent cyanobacterium should be respected in the choice of organisms for subsequent experiments.

4 Discussion

Cyanobacteria are an important phylum of bacteria regarding basic research and application. Ancestors of present cyanobacteria are the inventor of oxygenic photosynthesis and the origin of plastids. Members of the cyanobacterial subsection IV and V are among the most complex organisms known for the bacterial domain and have characteristics of multicellularity. Cyanobacteria play a significant role as fixer of atmospheric nitrogen and primary producers, making them an important factor for ocean's ecosystem and for the atmospheric CO_2 level. Thus, cyanobacteria are important objects for basic research. Furthermore, cyanobacteria got into focus for green biotechnology. Their photoautotroph lifestyle raises interest for the production of basic organic compounds as source for biofuels, bioplastics, or precursor for chemical industry. Also secondary metabolites of cyanobacteria are investigated for pharmaceutical applications (see also introduction).

For these basic and application aspects of cyanobacteria research, tools for genetic manipulation are extremely useful. While the number of sequenced genomes increases rapidly, the increase of new transformation protocols for so far genetic inaccessible cyanobacteria is slow and tough.

Research often focuses on naturally transformable strains of the order *Synechocystis* and *Synechococcus*, especially if genetic manipulation is required, for example for knock out mutants or heterologous overexpression of proteins. High efficiency and easy handling of natural transformation (NT) into the mentioned members of the Synechococcales is probably one of the main reasons, why they emerged as model organisms among cyanobacteria. Also members of heterocyst forming, filamentous cyanobacteria are important model organisms (for example in cell differentiation and nitrogen fixation). They are genetically accessible via conjugation or electroporation (EP; see also introduction).

Especially for application orientated research it is important to have a broad range of cyanobacteria accessible, because so far unexplored cyanobacteria may possess beneficial properties for biotechnology. Concerning basic research, a too strong focus on already established organisms may cause the missing of interesting aspects.

The genus *Phormidium* was so far not genetically accessible and the species *Phormidium lacuna* isolated by our workgroup shows promising properties for biotechnology (Nies *et al.*, 2017). This organism was chosen to establish a protocol for gene transfer and to characterize its potential in heterologous gene expression for biotechnology purpose. Transformation should be achieved by integration of the genetic information into the host's chromosome. This is considered more stable in contrast to use of an autonomous replicating plasmid. The chromosomal integration was also considered a save alternative in respect to later application in biotechnology with potentially unavoidable environmental exchange. A mobile genetic element like a plasmid might allow the exchange of genetic material, especially antibiotic resistance, with environmental bacteria more easily. Also chromosomal integration is less prone for losing the recombinant phenotype upon incubation without antibiotic pressure.

Gene transfer into other members of the order Oscillatoriales is already established with EP (Table 1 in the introduction). So this was considered the method of choice. Conjugation was regarded an alternative if attempts to establish gene transfer with EP would not result is transformants. Initially, NT was not considered a method for gene transfer, since it is discussed in literature often as a trait exclusive for only a small range of unicellular cyanobacteria. In fact, NT was so far only known for three genera of the order Synechococcales and for one *Microcystis* strain (Order: Chroococcales) in terms of heterologous gene expression. There is also one example of mutant complementation with extracellular DNA in the filamentous cyanobacterium *Nostoc muscorum* in the early 80s. However, no heterologous gene expression was demonstrated in this case.

Thus, the discovery of NT in *Phormidium lacuna* in this work is an important finding from different point of views. With a focused view on *Phormidium lacuna* itself, NT allows gene transfer with high efficiency and additionally the high kanamycin resistance enables fast segregation of the heterologous sequence in the genome. This makes it highly comfortable to perform recombinant work with this organism, enabling further biotechnology characterization, and it is also recommending *Phormidium lacuna* as a model organism for the Oscillatoriales, an important order of filamentous cyanobacteria without heterocysts (former classified as member of subsection III). In contrast, the most prominent member of the Oscillatoriales, *Arthrospira*, is transformable by EP, but the organism's restriction enzymes interfere with the transformation system (Toyomizu *et al.*, 2001, Jeamton *et al.*, 2017). Impact of nucleases was not monitored for *Phormidium lacuna*, but due to efficiency and convenience of the NT protocol, considerations of restriction enzymes are not essential in the first line. However, recombinant ethanol production could not be monitored in any transformant of *Phormidium lacuna*.

With a broader view on the finding of NT in *Phormidium lacuna*, the question arises if NT is a much more common trait among the cyanobacteria than generally thought. So far natural competence of cyanobacteria was known for members of the Synechococcales, one member of the Chroococcales and one member of the Nostocales. With *Phormidium lacuna*, also one member of the Oscillatoriales is known for NT by now. The four orders mentioned cover the big majority of all cyanobacteria known so far. Since for each of these orders at least one example for natural competence is known, it might be that NT is possible for much more cyanobacteria than the strains listed as naturally competent cyanobacteria (NCC) in Table 16. Homology based prediction in this work implicate that a broad spectrum of cyanobacteria is potentially naturally competent. If this could be verified experimentally, it would make various cyanobacteria genetically accessible, perhaps also in high efficiencies, since NT allows easy and uncomplicated gene transfer in *Phormidium lacuna* and other NCC. Since the establishment of transformation methods for a new cyanobacterium is still a challenging procedure (in which NT is normally not considered), it is a limiting step in characterizing new cyanobacteria in basic research and it also limits the use of a broader spectrum of cyanobacteria in recombinant green biotechnology. Therefore, it would be particularly helpful if based on the finding of this work, capability of NT could be found in various cyanobacteria.

In this section the findings of this work are reflected and set in relation to each other and to the scientific literature. Furthermore, possible future projects based on this work are discussed.

4.1 Protocol development

Protocol development was a central point of this work. Here, the influences of different factors are discussed, also in respect on how insight into this process of optimization can possibly be transferred to the investigation for NT in other cyanobacteria.

4.1.1 Natural transformation! But electroporation …?

With the detection of NT in *Phormidium lacuna* immediately the question arose if EP has a positive effect on transformation or if all successful transformations can be explained exclusively with NT. If so, EP would just have a negative effect on cell viability. The EP experiments in this work after the discovery of NT were meant to address this question and to characterize these two methods in direct comparison. Under 3.1.4, the central findings in comparison of NT and EP are listed briefly. Here, a more detailed analysis of this question is performed.

The differences in the EP protocol III and the NT protocol are quite low and consist mainly in the question if the sample is electrically pulsed or not. Thus, it has to be considered that NT can take place also during the EP protocol. It could be observed that negative effect of the electric pulse on cell viability increased with increasing field strength. The major difference between the protocols might finally be the impaired cell viability in the EP samples that explains the reduced transformation efficiency in comparison to NT (Figure 12, Figure 14, Figure 15).

However, beneficial effect of EP might be possible, despite its negative effect on cell viability. One indicator for support is that NT was not observed in a control without EP in 2016 for HE10JO. Also sample pulsed with only 1 kV cm^{-1} did not result in transformants (electronic supplement: transformation experiments). But both approaches would be more likely to result in transformants if only NT is the cause for transformants, since the negative effect of the pulse would be absent or smaller than for the frequently tested range of 2.5 – 5 kV cm^{-1}. However, both parameters were tested only once during the establishment of the protocol. Low transformation efficiencies with the EP protocols can also mean that potential transformation conditions were not monitored by chance. Also the conditions for NT tested in 2016 were different to the later ones and included a 3 h incubation step on ice of the mixture of concentrated cells and DNA. Later, it was demonstrated that prolonged incubation in water decrease cell viability (Figure 9, Table 8). Thus, the positive effect of no EP on cell viability was probably compensated by the negative effect of prolonged incubation in water in this case.

If EP did not have a negative effect on cell viability, one could conclude that if there was the additional effect of EP mediated transformation, one would expect the same transformation efficiency like for NT or even higher ones. But this is not the case. Reduced cell viability due to the electric pulse

is the most likely reason to the differences in transformation efficiency between NT and EP. Another approach to monitor a potential effect of EP was to test if EP enables transformants with lower amounts of DNA. In fact, EP protocol resulted in transformants with 0.3 µg plasmid, while 3 µg was the lower limit for NT (see electronic supplement: transformation experiments). But the number of approaches that addressed this question is too low to make a clear statement. It cannot be excluded that somewhere in this range is the limit for DNA uptake via natural competence and by chance the transformation worked in an approach, in which the sample was additionally electrically pulsed. To clarify this question, additional experiments are required.

After successful establishment of an efficient NT protocol the aspect of the influence of EP turned to lower priority. Although optimization of the NT protocol will most likely lead to further increase of transformation efficiency, the developed technique was considered sufficient for the subsequent creation of transformants for ethanol production.

To completely clarify if EP can cause transformants in *Phormidium lacuna*, the possibility of NT has to be excluded. Two washing steps were enough to remove free plasmid DNA and prevent NT (Table 11). However, this approach is probably not suitable for EP protocols, since cells are stressed after the electric pulse and additional washing steps will probably lower cell viability further. One possibility would be to use f/2 medium for washing after EP since negative effect of the washing steps before EP is mainly linked to the use of H_2O as washing medium (Table 8). But also here the impact of additional washing steps has to be investigated first. With the generation of knock out mutants deficient in NT, DNA uptake by natural competence can be excluded completely. A list of candidates can be found among the natural transformation factors (NTFs) in Table 13. Especially the Com-proteins would be interesting targets, since for Pil-proteins, RecA and DprA additional and important functions are known. For *Thermosynechococcus*, firstly NT was shown (Onai *et al.*, 2004) and secondarily the increased transformation rate of an EP protocol was described that does not, however, exclude NT (Iwai *et al.*, 2004). In contrast, EP does not show increased transformation efficiency in *Phormidium lacuna*. Also for *Thermosynechococcus* the additional influence of EP could be demonstrated since it promoted single and double recombination events, while during NT only double recombination events take place (Iwai *et al.*, 2004). Also in *Phormidium lacuna* only double recombination events were monitored (see also 3.1.1). Since during NT linear and single stranded DNA is taken up, double recombination is most likely, while during EP a functional plasmid enters the cell and both recombination events are generally possible. The differentiation between single and double recombination events in *Phormidium lacuna* bases on PCR results (Figure 8 in the result section, Figure 20 in the supplement). This is regarded sufficient but southern blot analysis would be a more sophisticated way to prove this.

Nevertheless, most findings support an exclusive role of NT also in EP protocols. The exclusive finding of double recombination events (see above) is one strong indication. Another important indication is the time point of NT. Table 11 shows that DNA uptake takes place in suspension culture

or even on the agar plates, a time point very distinct (up to 18 h, upper limit not demonstrated) from the time point of EP. Therefore, EP mediated transformation is hardly conceivable.

Also the restoration of transformability during the EP protocol development give strong hint for NT in retro-perspective: The two changes performed, the higher OD_{750} range and the obviation of condensation, were probably crucial for restoration transformability (OD_{750}: optical density at 750 nm). For several bacteria it is known that competence is dependent of the growth phase. Since OD_{750} does not only indicate cell density but also the growth phase, it is more likely that *Phormidium lacuna* takes up DNA from the environment more efficiently in later growth phases. But also just a higher number of transformable cells might increase transformability. But OD_{750} of 0.28 and 0.3 was tested before the restoration of transformability in 2017 for HE10DO without resulting in transformants (electronic supplement: transformation experiments). Thus, the second change, which most likely restored transformability (see below), had probably a bigger influence. The evidence for impact of growth phase on transformability requires further experiments, which also distinguishes between cell density and growth phase.

A further advantage of the narrowed and higher OD_{750} range is that cells seemed to tolerate EP better. Higher cell number and better viability improved handling and lowered variations in cell propagation during protocol optimization. OD_{750} is an indicator for cell density but also for growth phase. Growth phase was considered an important factor from the beginning on, but only with a bigger number of transformation events a closer correlation of the OD_{750} and transformability could be monitored. Once the optimal OD_{750} range became obvious (Table 9), inoculation and cultivation of bacterial cultures was performed to reach this range.

Even though the impact of condensation events seems a bit obscure on the first view, it makes sense if one considers that it did not negatively affect cell viability. Differences in the propagation of *Phormidium lacuna* or other cyanobacteria due to condensation events were not noticed. Therefore, condensation was not considered from an EP point of view at first. But the condensation events also alter the conditions during the possible time point of DNA uptake in the 2 d cultivation between the washing steps and transfer to agar plates (see 3.1.3). Hence, the change of cultivation conditions led probably to an increased efficiency of NT. The lack of condensation might have changed the illumination of the cells or it correlates with temperature changes. For the influence of growth phase and culture condition on NT in other cyanobacteria also consider 4.2.1.

In contrast, a possibly positive effect of EDTA to enhance DNA uptake during EP, maybe through cell wall destabilization or nuclease inhibition (Gray & Wilkinson, 1965, Cao *et al.*, 1999, Alakomi *et al.*, 2006), could not be confirmed. Furthermore, EDTA had to be lowered from the initial protocol's concentration to a less harmful concentration to not inhibit cell viability, especially of the HE10DO strain, for which NT exclusively was demonstrated. Also other approaches to increase DNA uptake via EP did not show positive effects, while optimization in cell viability enhanced transformation efficiency (see also 3.1.2). This also supports an exclusive role of NT in the transformation of

Phormidium lacuna, since DNA uptake is active and autonomous process in natural competence. Thus, strategies to increase DNA uptake via EP (for instance possible cell wall destabilization with EDTA) would not show any result and would be even contraproductive.

Experiments with the autonomous replicating plasmid pVZ321 were only evaluable before restoration of transformability and did not result into transformants. However, it fits the finding of NT that replicating plasmids are harder to transform than plasmid for homologous recombination. DNA enters the cell single stranded. This is sufficient for recombination into the chromosome, but for restoration of a replicating plasmid probably more than one single stranded plasmid copy has to be present in the cell. However, the model organisms *Synechococcus elongatus* PCC 7942 and *Synechocystis* sp. PCC 6803 can also be naturally transformed by self-replicating plasmids (Chen *et al.*, 2016, Liu & Pakrasi, 2018).

In summary, in the comparison of the pros and cons there is a clear preference for NT as exclusive factor permitting transformation in all protocols. Also, freely adapted from Ockham's razor, it seems to be more reasonable to find just one new transformation possibility into an organism; especially when the protocol of the one method, EP, enables the other method, NT, but with declined efficiency that is logically explainable through reduced cell viability.

From the results supportive effects of EP or EDTA on transformation cannot be supported but also not be excluded completely. Despite their negative effect on cell viability, EP or EDTA could be helpful in overcoming factors that negatively affect NT (see 4.2.2).

Homogenization showed once to be enough to enable NT in HE10DO (see Figure 14 and electronic supplement: transformation experiments). This demonstrates that the requirements for NT might not be that complex and that other steps of the protocol could be reconsidered, since it is still strongly influenced by the requirements for EP. Especially the washing steps with H_2O that showed to decrease cell viability (Figure 9, Table 8) can be reconsidered. The washing steps may be also supportive for the transformation of *Phormidium lacuna* (see 4.2.2). But one could supplement H_2O with artificial seawater or f/2 medium. H_2O was used in the washing procedure to remove salts prior to EP but this is not necessary in a NT protocol. Washing with f/2 medium does not have a considerably impact on cell viability (Table 8). The determination of the time point of NT and the restoration of transformability further illustrate that varying the conditions in this protocol phase might have influence of transformation efficiency. This is further discussed under 4.2, where the finding of NT in *Phormidium lacuna* is set into relation to the literature.

In retrospect, one should have tested for NT more often as a control. Probably, it would have been detected much earlier and more frequently. However, the negative results for NT in 2016 matched perfectly with the expectations from literature: 1) NT is not documented for filamentous cyanobacteria, with one exception for the Nostocales, and several EP protocols for other representatives of the Oscillatoriales exist. 2) Numerous conditions were tested during protocol

optimization and cultivation space was limited. Therefore controls considered unlikely to be necessary were excluded.

4.1.2 Further considerations for protocol development

Main object of the protocol optimization was the establishment of an efficient and reliable transformation protocol. This was finally achieved with the finding of NT for HE10DO. The effect of the different conditions during protocol development was critical for the optimization, but for a reliable protocol it was not necessary to evaluate every factor's impact in a significant manner. For some factors, this is only possible with great effort or not possible at all.

Calculation of transformation rates (number of transformation events in one approach in dependency of used DNA amount) was not the major objective during protocol development, since many approaches did not result in transformants at all. It was more important to confine the range of conditions that allow transformation in most approaches (high transformation efficiency) irrespective of the transformation rate. With NT, most approaches result in transformants with the plasmid 7-K. This high transformation efficiency was sufficient for the generation of transformants for ethanol assays and thus protocol optimization stopped with the discovery of NT in *Phormidium lacuna*. However, for the further optimization of the NT protocol it is important to enable reliable and precise statements on transformation rates.

For several factors tested in this study, it was not possible to evaluate their influence on transformation efficiency or there were contradictory results, like for the influence of homologous sequence length on transformation efficiency (see below). This reveals a limitation in the comparative analysis of multiple transformation experiments under partial different conditions and at different time points. The central limitation of the overall analysis of transformation efficiencies under 3.1 is that *Phormidium lacuna* does not form colonies. While for a colony forming bacterium, transformation rate can be determined for every single approach quantitatively, for the filamentous cyanobacterium *Phormidium lacuna* transformation is a binary yes/no event. Thus, transformation efficiency can only be calculated in the comparison of various approaches with comparable conditions. Taken this into account that the fluctuations between the different experiments are incorporated into the calculation this limits the precision of the calculation further. For several periods of this work the monitoring of transformation by binary yes/no event was sufficient, since it was first important to enable transformation of *Phormidium lacuna* at all. But for the further optimization of transformation protocols, especially for NT protocol with its high transformation efficiencies (ca. 90%), a new way to monitor transformation rates is required.

A possible way to handle this limitation of *Phormidium lacuna* is to make dilution series when transferring the samples on plates. However, this strategy increases the amount of agar plates dramatically and this step was not executed so far due to limitations in resources and work force. Also homogenization would probably be required to allow a very precise distribution of the cells. At least

with Ultraturrax, the standard device, this would be challenging due to the required amount Ultraturrax wands. Homogenization by sonication would be a notable alternative worth testing. For further optimization of the NT protocol and monitoring the influence of the single parameters a more precise detection of transformation rate is advisable despite bigger efforts in protocol execution. In this work it was tried to calculate transformation rates for single plates via identification of the starting point of filament's growth for single transformants. However, this approach needs regular and intensive microscopy for every single plate and the approach's validity is restricted by the motility of transformants. Therefore, dilution series would be a promising strategy to test. Additionally, immobilization of *Phormidium lacuna* cells inside agar medium might be a promising strategy. Also varying the media composition might have a negative impact on the motility of *Phormidium lacuna* on agar plates and could lead to the formation of colony like cell propagation. These strategies are investigated presently in our workgroup by Nora Weber (master thesis)

Viability and transformation efficiencies differed between experiments and also now this is not understood completely. For example, the strong viability loss in experiments in late 2016 cannot be explained entirely. Potential factors with systematic impact are considered, like changes in the water quality or in the culture room conditions without convincing results. Smaller fluctuations between subsequent experiments made it difficult to distinguish if changes in viability or transformability are caused by a tested parameter or if it is just a correlation. The influence of multiple factors on transformability, the limited number of samples per experiment (maximal sixteen, including controls), and the long time span until evaluation of the experiment restricted even the possibility for a systematic analysis of the influence of the major factors. Therefore, some judgments of certain influencing factors are rather experience based assumptions than validated results.

For other factors, only the overall comparison finally reveals if they have a beneficial impact or not. In list of transformation experiments (electronic supplement), the positive impact of a higher OD_{750} range can be derived, even though the first transformation 2017 indicated first a lower OD_{750} (an example for misleading correlation). EDTA, on the over hand, was long time considered to maybe increase transformability, but this assumption could be neglected in three comparative experiments and also by an overall comparison (Table 10, Figure 11). For other factors, the data set is not big enough for significant evaluation but nevertheless possible trends can be indicated, like for the influence of insert size. Potentially reduced transformation efficiency with increasing insert size, which in accordance to literature for other bacteria (Kung *et al.*, 2013), has implications on further steps to biotechnological application. For the introduction of recombinant pathways in general a selection marker and a least one additional open reading frame (ORF) have to be inserted into the genome of *Phormidium lacuna*. Thus, size of the non-homologous insert for the generation of producer strains will be bigger than the one of the frequently tested plasmids in this work (7-K plasmid and its variations). Restrictions in this respect could be faced by further optimization of the transformation protocol (see below).

The influence of homologous sequence length stays elusive. In the direct comparison of 7-K and 7-K:2k in Table 10, longer homologous sequences on the plasmid seem to be beneficial. This is consistent with the literature, since for *Synechocystis* sp. PCC 6803 the positive effect of longer homologous sequences was already demonstrated (Zang *et al.*, 2007). In an overall comparison (Figure 14) for *Phormidium lacuna*, this cannot be demonstrated clearly. Possibly, longer homologous sequences are also beneficial for transformation efficiency in general in *Phormidium lacuna*, but this cannot be demonstrated during EP. Probably, the effect of impaired cell viability overlaid this effect. For NT, the data set is too small for an overall comparison in this respect.

The transformability of HE10JO, the strain with the sequenced genome, could not be reestablished after the loss of viability in late 2016. The main reason is probably the contamination with *Marivirga* that could not separated from HE10JO during isolation. During normal conditions, HE10JO propagation is not impaired by this organism. But under selective pressure or in stationary phase after prolonged cultivation, strong propagation of *Marivirga* can be observed. This decreases viability of HE10JO and it is concluded that either stressed HE10JO is not able to defend against *Marivirga* or that a former commensal symbiosis turns into a predator prey relationship. Thus, the contamination of the HE10JO stock culture was an additional factor to consider during multifactorial protocol optimization. Therefore and because of the successful transformation of HE10DO in 2017, protocol optimization concentrated on the strain HE10DO. Since EP and NT protocols are optimized for axenic HE10DO strain, this may be one reason why the protocols could not be transferred to non-axenic HE10JO one-by-one.

Nevertheless, it is assumed that also NT was relevant during the transformation of HE10JO in 2016, although it cannot be demonstrated explicitly until transformability is restored for this strain. The very close relation of HE10JO and HE10DO, the same transformation efficiency of the EP protocol I for HE10JO and the EP protocol III for HE10DO (Figure 12), and the possibility to transform HE10JO, HE10DO and GI09CO (bachelor thesis of Finn Zaiß) with the EP protocol I are indicative all together for the same transformation mechanism in both protocols, namely NT. Several approaches were tried to remove *Marivirga* from the HE10JO stock culture. The different strains of *Phormidium lacuna* were isolated from environmental samples by serial dilution by other members of our workgroup. This was successful for some strains like HE10DO and GI09CO, but for other strains like HE10JO the *Marivirga* contamination could not be removed the same way. This supported the assumption that *Marivirga* is somehow positive influenced by HE10JO cells (living or dead). Further, the abilities of *Phormidium lacuna* to move on surfaces or to tolerate higher temperatures (Nies *et al.*, 2017) as well as filtration were tested by Kevin Schulz (bachelor thesis) in order to achieve separation of HE10JO from *Marivirga* but without result. Also the use of different antibiotics to remove *Marivirga* or inhibit its growth did not show convincing results. As other members of the genus *Marivirga* are known to harbor several antibiotic resistances (Pagani *et al.*, 2011), a more sophisticated approach of isolation

by antibiotic pressure might be the most promising approach with selection of promising antibiotics and the combination of differing cultivation conditions.

Another possibility is to use the HE10JO transformants from 2016. *Marivirga* could only tolerate minor kanamycin concentration but not higher concentration in the range of several milligrams per milliliter (Figure 16). Thus, the HE10JO transformants are axenic. To profit from this transformants, the use of a new selection marker is necessary. One could use the same integration site and knockout the *kanR* gene. Or one could also use a different locus for integration into the genome. For both cases more research is necessary. But both points are discussed for further optimization of the transformation system of *Phormidium lacuna* anyhow (see 4.3). Thus, with further development of the molecular tools for *Phormidium lacuna* HE10JO transformants can be used to work axenic with this strain. Before this, it has to be demonstrated whether the axenic HE10JO transformants are transformable with the present or a further optimized NT protocol.

The fast segregation of recombinant sequences in transformants of *Phormidium lacuna* is a significant advantage. In fact, for transformants of other cyanobacteria it is reported that antibiotics have to be raised in small steps over longer timer periods and that the selection of homozygous transformants can be very time consuming (Behler *et al.*, 2018). The high kanamycin resistance in *Phormidium lacuna* most likely enables the fast segregation: Even though not fully segregated transformants can tolerate high kanamycin concentration, cells with fully segregated genome have a significant advantage in propagation and dominate the bacterial culture very fast. This might also explain the dying of transformants on prolonged incubation on agar plates with 120 µg/ml kanamycin (Figure 10) even though in suspension culture transformants can survive on much higher concentrations (Figure 16): On agar plates cell propagation rate is much lower than in suspension culture. Through cell division and the uneven distribution of wild type (WT) and recombinant chromosomes in the daughter cells the selection marker can segregate in the genome. Thus, on agar plates the segregation rate is probably too low and also 120 µg/ml kanamycin can cause complications for transformants over time. Notably, there are also differences in the medium between selective agar plates (f/2) and selective suspension culture ($f/2^+$). In $f/2^+$ suspension culture with tenfold increased nitrate and phosphate concentration, *Phormidium lacuna* cultures can be cultivated for much longer time periods than in f/2 suspension culture (f/2: maximal 2 weeks, $f/2^+$: several months). An alternative reason for the growth arrest on f/2 agar plates with 120 µg/ml kanamycin might be that the cells are additional stressed by consumption of nitrogen and phosphate sources in the medium during prolonged cultivation. Thus, they are more vulnerable to kanamycin.

For the high antibiotic resistance of HE10JO and HE10DO transformants no corresponding example was found in literature. However, in environmental samples of *Enterococci*, also high kanamycin resistances in the range of 2 mg/ml were observed (Dada *et al.*, 2013). Compared to transformants of other species in our lab, comparable high resistance in *Escherichia coli* or *Synechocystis* sp. PCC 6803 cannot be reported.

The high kanamycin resistance of *Phormidium lacuna* transformants is not fully understood: Probably high expression of the selection marker is not the cause, since no heterologous ethanol production could be detected (see 3.3). Therefore, it is more likely that low to moderate *kanR* gene product mediated resistance is combined with an endogenous resistance or tolerance towards kanamycin or antibiotics in general. This is supported by the long time phases required for selection on plates, which indicate that *Phormidium lacuna* can survive quite a long time on moderate kanamycin concentrations. This could indicate a natural resistance or at least tolerance mechanism towards kanamycin. The addition of a heterologous resistance mechanism may lead to a multiplication of resistance level, also with a moderate expression of the selection marker. Potential aminoglycoside resistance mechanisms are reviewed in literature (Garneau-Tsodikova & Labby, 2016).

4.2 Why natural transformation – possible strategies for optimization of NT

The finding of NT in *Phormidium lacuna* raised the question if natural competence is a much more common trait among the cyanobacteria than considered until now by the scientific community. For establishment of an efficient transformation protocol, the effect of the single factors is not important during the protocol, as long as the protocol works efficiently. Thus, after the establishment of a reliable transformation protocol by NT focus was on the generation of transformants for recombinant ethanol production. However, in the question why NT in found for HE10DO with the actual NT protocol (and most likely was also found for HE10JO, GI09CO and HE10DO with EP protocol I with lower efficiency) but natural competence is not documented for any other member of the Oscillatoriales, a deeper understanding of NT is required. This is especially true if based on the finding of this work, the distribution of natural competence among the cyanobacteria should be investigated. The bioinformatics data under 3.4 indicate that the majority of cyanobacteria could be capable of NT. So it is important to know, which are the crucial factors enabling NT in HE10DO and if these might also be essential for other cyanobacteria.

Three central points are essential for natural competence in cyanobacteria: 1) Complete machinery for DNA uptake meaning that all relevant proteins are expressed and functional. 2) DNA integrity during the whole process. 3) Contact between the type IV pilus and DNA.

1) Under 3.4, homologs of the NTFs of NCC are found in the genome of many cyanobacteria. This is a requirement for natural competence, but not sufficient. Natural competence can be dependent on the growth phase or other environmental factors, possibly due to gene expression or other regulations.
2) Under 3.1, it was mentioned that DNA quality can be critical during transformation procedure. Furthermore, plasmid DNA can be degraded by nucleases of the cyanobacterium. There are examples that strong nuclease activity interferes with transformation, like for EP protocols of *Arthrospira* (Toyomizu *et al.*, 2001, Jeamton *et al.*, 2017). It is also possible that a certain nuclease activity is necessary for natural competence. DNA is supplied as plasmid during NT

of *Phormidium lacuna*, but it enters the cell as a linear, single strand molecule (see Figure 4 in the introduction). Thus, plasmid has to be cut and one strand has to be degraded, but the time point(s) are not known by now.

3) Cyanobacteria build extracellular polymeric substances (EPS), mainly exopolysaccharides. This is also true for *Phormidium lacuna* and can be seen in moving tracks on agar plates (Figure 10 C in 3.1.2). EPS build an extracellular matrix labeled sheath, capsule or mucilage, depending on the structure. Extracellular nucleases can be embedded in this matrix (Stucken *et al.*, 2013). EPS may hamper the contact between pilus and plasmid sterically, by charge, or enzymatically through embedded nucleases.

4.2.1 Natural competence in cyanobacteria – DNA uptake machinery

Concerning functional DNA uptake machinery, so far no growth phase dependent expression studies of NTFs were performed in cyanobacteria. However, for *Synechocystis* sp. PCC 6803 it is known that rate of NT is growth phase dependent (Zang *et al.*, 2007) as well as for *Thermosynechococcus elongatus* BP-1 (Iwai *et al.*, 2004) and *Synechococcus* sp. PCC 7002 (Stevens & Porter, 1980). Although there are differences between the strains, all have in common that in stationary phase NT rate was at the lowest level but still present. For HE10DO, OD_{750} range from 0.25 – 0.35 showed the best results for transformation, fitting the results of other cyanobacteria. Taken together, growth phase is a central point to consider if testing for NT in other cyanobacteria or to characterize NT further in HE10DO. In contrast, for some other bacteria natural competence is only induced by certain factors. For example, in *Vibrio cholera* natural competence is only found with the induction by chitin (Meibom *et al.*, 2005).

Possibly also other factors exist that would influence natural competence in cyanobacteria. To have an idea what factors would be possible, one should consider the possible purpose of natural competence in cyanobacteria. Three possible reasons are discussed in literature for bacteria in general that are not mutually exclusive: Nutrition, DNA repair, recombination. Many examples in literature exist for reviews of this question. For the following discussion several reviews were considered and can be looked up for a deeper understanding of the topic (Johnsborg *et al.*, 2007, Seitz & Blokesch, 2013, Mell & Redfield, 2014, Blokesch, 2016). For cyanobacteria however, the indications of a specific purpose of natural competence are poor and NT is mostly considered from a descriptive, mechanistic, and applied point of view. For *Synechocystis* sp. PCC 6803 the influence of NTFs on transformability was investigated (Table 13). The applied perspective is also true for most parts of this work. A systematic approach of distribution of NT in the phylum would be a sophisticated strategy to enhance the cyanobacterial research in general. A deeper understanding of the need of natural competence in cyanobacteria might support these efforts.

However, all three purposes listed above have their justification also for cyanobacteria:

DNA as food source is discussed controversially for bacteria in general: A direct, evolutionary advantage is obvious in contrast of the long-term influence of potential beneficial recombination events and there is supportive information for some bacteria. For photoautotroph cyanobacteria however, DNA uptake might not be relevant in perspective of carbon and energy source. But DNA may be a source of nitrogen or phosphorous. The increase of the nitrate and phosphate concentration by a factor of ten (f/2 media in comparison to $f/2^{+}$) improved growth behavior in *Phormidium lacuna*, indicating the importance of these two compounds. For the increase of NT possibly the other way is promising: The starvation of phosphate and/or nitrate during the time span of NT might increase the uptake of DNA. Homologous recombination would be a byproduct in this perspective.

DNA uptake for **repair** purposes makes sense if DNA of the same or closely related species is incorporated. This is known for other bacteria: For example *Haemophilus influenza* can distinguish between foreign and species specific DNA based on an 11 bp motive (Danner *et al.*, 1980). As photoautotrophs cyanobacteria are exposed to UV irradiation and reactive oxygen species, which both have mutagenic effects. Natural competence could be a strategy to repair DNA damages with identical or very similar sequences via homologous recombination. Thus, natural competence might be influenced by illumination (not necessarily by DNA damage or UV light). The polyploid lifestyle of many cyanobacteria is also most likely a strategy against DNA damages (see also 1.2.2) and natural competence would be another aspect of DNA repair. The correlation between restoration of transformability and condensation events (that also possibly altered illumination of HE10DO during NT) is an indication that this might be also relevant for *Phormidium lacuna*. For *Synechocystis* sp. PCC 6803 it was also shown that NT is dependent on illumination (Zang *et al.*, 2007). Alteration of illumination during the time point of NT is an approach to address this question experimentally for *Phormidium lacuna*.

Natural competence seems to be an universal or at least very common trait among bacteria and is also described for archaea (Johnsborg *et al.*, 2007). This implicates that much more bacteria, including cyanobacteria, could be naturally transformable, but the relevant conditions are unknown. The broad distribution of NT among the bacteria is most likely due to a universal function being maintained in this domain of life. NT is considered one of the three major mechanisms (together with conjugation and transduction) behind **horizontal gene transfer** and a counterpart for sexual DNA recombination of eukaryotes. It allows the fast and widespread distribution on beneficial mutations and traits, also across phylogenetic borders. For a bacterium that experiences strong variations of environmental factors like for cyanobacteria (illumination, mineral nutrient limitation, salt conditions, temperature, grazer, pathogens) an effective acquisition of beneficial traits by a possible inducible uptake system would be reasonable. Factors inducing natural competence, including environmental factors, are known for gram positive and negative model organisms for NT (Seitz & Blokesch, 2013, Blokesch, 2016).

Taken together, the conditions during the time point of NT are likely to have influence on the transformation rate. Factors like growth phase, illumination, media composition (salt, nitrogen and phosphate content) and temperature are all possible factors influencing DNA uptake. So far there is no evidence for bacteria that extracellular DNA itself can induce natural competence (Johnsborg *et al.*, 2007, Mell & Redfield, 2014). For *Synechocystis* sp. PCC 6803, it was additionally shown that the time span for NT has a big impact on transformation rate (Zang *et al.*, 2007). In comparison, it could be observed for HE10DO that DNA addition at various time points leads to transformants (Table 11). It would be a promising approach to monitor transformation rates of *Phormidium lacuna* in dependency of incubation time with plasmid DNA. Time point of DNA uptake was narrowed down to suspension culture even though not the full 2 d are required or it takes even place on agar plates. Possibly, selective pressure on agar plates may have influence on DNA uptake. To test this, a variation of experiment has to be performed and DNA has to be added not before cells are transferred on agar plates.

4.2.2 Natural competence in cyanobacteria – natural barriers

Beside the potential of the bacterium for DNA uptake also the integrity of the DNA and the contact between DNA and the type IV pilus are important for NT. Nucleases and EPS play an important role in the perspective. They are handled together since possible methodical responses are similar in some cases. As mentioned, the negative effect of nucleases is described in several transformation protocols of cyanobacteria. Various types of nucleases are known and different kind of classification exist: Exo- and endonucleases, ribo- or desoxyribonucleases, double strand or single strand specific nucleases, sequence specific or sequence independent nucleases. Restriction endonucleases cut sequence specific double stranded DNA and are abbreviated as restriction enzymes. They are used by bacteria to distinguish between endogenic and foreign DNA. Methylases can methylate sequence specific recognition sites and thus grant protection from the respective restriction enzyme. Methylation of restriction sites showed significant increase of transformation rate in cyanobacteria, for instance for *Anabaena* sp. M131 (Thiel & Poo, 1989). Also on the genome of *Phormidium lacuna* restriction enzymes are encoded (Nies *et al.*, 2017).

DNA enters the cell as a single strand molecule during NT. Thus, up-taken DNA is possibly protected by restriction enzymes, since they are generally known to cut double stranded DNA. There is also report that single stranded DNA can be used a substrate in vitro (Horiuchi & Zinder, 1975, Nishigaki *et al.*, 1985). However, it is not known if this is biologically relevant since the single stranded DNA in the cytoplasm is associated with single strand binding proteins, DprA, and RecA. Extracellular restriction enzymes and other nuclease, however, have to be considered anyhow.

The restriction enzymes in the HE10JO genome can be grouped in three annotations: Unidentified restriction enzyme, identified but unknown recognition sequence, identified. Also Methylases were considered since they provide protection against the respective nucleases for the host, which thus were

also considered probably present. Seven recognition sequences could be identified by this (Table 17). All of them except the one of NspV are found on the 7-Kn:2k plasmid, some of them multiple times. If also the corresponding nuclease to the methylase DsaV is present, its recognition site on 7-Kn:2k would be blocked by the *Escherichia coli* DH5alpha Dcm methylase probably. The listed restriction enzymes do not seem to have a crucial effect on transformation of *Phormidium lacuna*. However, this should be tested for further optimization. Plasmid could be incubated with *Phormidium lacuna* culture, cell lysate, or culture supernatant can be investigated on agarose gels in dilution series for DNA integrity. Mutation of detected cleavage sites or coexpression of the respective methylase in the *Escherichia coli* strain for plasmid preparation would be possible further approaches to answer on possible restriction events. Possibly, this is a promising was to increase the efficiency of the NT protocol.

Common restriction enzymes in cyanobacteria (Lyra *et al.*, 2000) are AvaI (5'-C|YCCRG-3'), AvaII (5'-G|GWCC-3') and AsuII (5'-TT|CGAA-3') and corresponding isoschizomers can be found for AvaII and AsuII in *Phormidium lacuna* (Table 17). DNA motifs recognized by restriction enzymes are predicted based on genome data for a broad range of cyanobacteria in literature (Stucken *et al.*, 2013). Nuclease activity in the culture medium can probably not only reduce transformation efficiency but also influence factors like DNA amount required for transformation or the optimal time span for transformation.

Table 17: Annotated restriction enzymes and site specific DNA methylases with known recognition site (Nies *et al.*, 2017). Commercial isoschizomers provided by NEB (USA) are listed. The annotated function of the listed enzymes is listed. It is distinguished between the restriction enzyme and the methylase. If the methylase was annotated, it was assumed that also the respective restriction enzyme is present without being annotated. K = G or T; M = A or C; R = A or G; W = A or T; Y = C or T.

Name	Recognition site	Annotation code	Putative function	Isoschizomers
AccI	5'-GT|MKAC-3'	phl#1080	Methylase	AccI
BsuBI	5'-CTGCA|G-3'	phl#3108	Nuclease	PstI
DsaV	5'-|CCWGG-3'	phl#2542, phl#2543	Methylase	PspGI
HgiBI	5'-G|GWCC-3'	phl#3829	Methylase	AvaII
HgiCI	5'-G|GYRCC-3'	phl#443	Nuclease	BanHI
NspV	5'-TT|CGAA-3'	phl#3313	Methylase	BstBI
PaeR7I	5'-C|TCGAG-3'	phl#968	Methylase	XhoI

A correlation between transformation rate and EPS level was so far not demonstrated for cyanobacteria. However, the EPS level for several, natural transformable model organism is low in comparison to other cyanobacteria and it is generally influenced by environmental conditions (Stucken *et al.*, 2013). There are different criteria used to classify exopolysaccharides in reviews (Pereira *et al.*, 2009, Rossi & De Philippis, 2015, Delattre *et al.*, 2016), for example if they are homopolysaccharides or heteropolysaccharides, or if they are covalently linked to the cell surface, released into the

environment, or somehow attached to the cells, for instance by charge. Cyanobacterial EPS can contain uronic acid and sulfate groups, giving them an anionic nature and an affinity to metal ions, while other EPS are characterized hydrophobic to a certain extent due to organic substitutes (Pereira *et al.*, 2009). Both properties may hamper DNA-cell contact.

EPS are considered to protect cells from unbeneficial environmental conditions like dehydration, UV stress, and phagocytosis but may also allow adherence growth on surfaces (Pereira *et al.*, 2009, Rossi & De Philippis, 2015). As primary producers, cyanobacteria are often first colonizers and their EPSs can contribute main parts to the matrix of microbial biolayers (Rossi & De Philippis, 2015). Due to their diverse functions, several parameter influence the production of EPS like the culture media (especially nitrogen and phosphorous), light, and temperature (Pereira *et al.*, 2009, Delattre *et al.*, 2016). Also *Phormidium lacuna* shows frequently adherent growth in suspension culture, especially in early growth phase. However, this is a side observation and was not investigated systematically, but it underlines the relevance of EPS for *Phormidium lacuna*.

While the negative effect of nucleases on NT is documented, the negative effect of EPS is just reasonably suggested (Stucken *et al.*, 2013). For both factors, the transformation protocols of *Phormidium lacuna* have a possible counteraction: Homogenization, necessary to determine culture's cell density, is well tolerated well by *Phormidium lacuna* but is nevertheless a harsh, mechanical treatment. It probably detaches EPS from the cell surface. Subsequent centrifugation and washing procedure removes probably parts of the extracellular matrix together with embedded as well as free floating nucleases. Thus, the pretreatment of *Phormidium lacuna* most likely reduces the negative effect of nucleases and EPSs.

Minimal pretreatment for NT, which was tested, is homogenization and cell concentrating with one centrifugation step (electronic supplement: transformation experiments). Addition of 7-Kn:2k resulted in transformants without additional washing steps. This did not work for the larger plasmid 7-P-A-K:2k. Since this was only tested in one experiment for each plasmid (7-Kn:2k: two approaches, 7-P-A-K:2k: one approach), this difference is explainable with the reduced transformation efficiency for longer sequences (Figure 13). Homogenization was the minimal treatment tested, because of OD_{750} measurement and because it is a prerequisite for comparable handling of *Phormidium lacuna* samples. To verify possible positive effects of homogenization on NT, one also has to test untreated samples in comparison. Also the association of EPS to cell surface can be examined depending on homogenization and washing steps by staining with India ink (Rossi & De Philippis, 2015). If the beneficial effect on NT is confirmed, homogenization with Ultraturrax is possibly easily transferable on other cyanobacteria. *Phormidium lacuna* can also be homogenized by ultrasound, but this was not tested in a transformation protocol so far. This would be another method to test. Ultrasound treatment in combination with Ultraturrax would also be interesting, since ultrasonic treatment produces shorter filaments (bachelor thesis of Dominic Fink), while Ultraturrax treatment breaks big cell aggregates more efficiently.

An observation challenging the possible effect of extracellular nucleases and EPS is the time point of NT (Table 11). Addition of DNA 18 h after homogenization and washing also results in transformants. Thus, cells have a quite long time period to regenerate their extracellular matrix to an unknown extent. Homogenization may still be beneficial because it loosens cell aggregates and shortens filament lengths and thus increases the cell surface accessible for DNA cell contact. Since 18 h allows only one to two cell divisions, the possible benefit certainly remains.

If homogenization shows effect on transformation efficiency as well as EPS cell association, then this would be a strong confirmation of a negative effect of nucleases or EPS or both on NT in *Phormidium lacuna*. Variation of homogenization conditions could then be considered for optimization, for example in respect of intensity, method (ultrasound or mechanically), or time point (also during cultivation or before transfer to agar plate).

Also other and former parts of the transformation protocols could be reconsidered under this perspective. The often negatively charged EPS are stabilized with divalent cations, like Mg^{2+} and Ca^{2+} (Rossi & De Philippis, 2015). Also for nuclease activity, divalent cations are required (Cao *et al.*, 1999). Since environmental conditions are already known to have effect on EPS production in cyanobacteria (see above), variation of the medium could be considered at different protocol steps, like reduced Mg^{2+} or Ca^{2+} concentrations. Also the washing of the cells (if proven beneficial) could be done by medium (standard composition or without certain compounds) or other solutions instead of water. Water alone showed to have negative effect on cell viability (Table 8) and removal of salt is just a remnant of EP conditions. EDTA, which complexes divalent cations, did not show to be beneficial in EP protocols like mentioned already. However, it was never tested in NT protocol without EP. Also the time point of EDTA treatment may be altered. EDTA (2 mM) in BG11 media increased transformation rate in *Synechocystis* sp. PCC 6803 by 23% (Zang *et al.*, 2007). Actually, EDTA is already contained in f/2 medium, but in much lower concentration. Since f/2 medium is sea water medium in contrast to BG11, probably even higher EDTA concentration would be required. 2 mM EDTA already caused viability losses for HE10DO during washing procedure, but in combination with medium the effect of EDTA was not tested. If EDTA would be used in further experiments, a precise balance between concentration, incubation time, and cell viability would probably be required. Anyway, previous indication of EPS influence on NT should be evaluated first.

Since association of non-covalently attached EPS relies on charge, also a positive effect of EP would be explainable without causing transformation directly. The electric pulse may help to re-orientate and loose the association of EPS with cell surface. For HE10DO NT is efficient enough to not rely on EP, but perhaps for other organisms this would be a possibility to consider. Actually, for some of the CNT^{+} or their close relatives transformation protocols via EP exist (CNT: candidates to test for natural transformation). Since the discovery of NT in *Phormidium lacuna* is based on the development of an EP protocol, the mechanism for transformation should be reconfirmed for these organisms (especially if the protocol is similar to the EP protocol I or EP protocol III). This is not meant as a challenge of

published results, but EP may have more a supporting impact but NT could be the crucial principle in some cases.

For three orders of cyanobacteria natural competence is already described: Synechococcales, Chroococcales, and Nostocales. In the intention to optimize NT in *Phormidium lacuna* and to investigate the distribution of natural competence for additional cyanobacteria, it is important to compare those already existing protocols and figure out what they have in common. Protocols of *Synechococcus* sp. PCC 7002 (Stevens & Porter, 1980, Ruffing, 2014), *Thermosynechococcus elongatus* BP-1 (Iwai *et al.*, 2004, Onai *et al.*, 2004), *Synechocystis* sp. PCC 6803 (Grigorieva & Shestakov, 1982, Zang *et al.*, 2007), *Synechococcus elongatus* PCC 7942 (Shestakov & Khyen, 1970, Kinney *et al.*, 2012, GeneArt Synechococcus Engineering Kit, Invitrogen, USA), *Microcystis aeruginosa* PCC 7806 (Dittmann *et al.*, 1997), *Nostoc muscorum* (Trehan & Sinha, 1982) were considered: Early discovery of natural competence (until 1982) relied on mutant complementation or acquisition of antibiotic resistance based on the uptake of genomic DNA of closely related strains. For all examples except *Nostoc muscorum* the genomic integration of a recombinant selection marker was demonstrated at later time points. All protocols have in common that cells and DNA are incubated for a specific time span that varied for the organisms. The time span showed to be crucial for *Synechocystis* sp. PCC 6803 as well as illumination (Zang *et al.*, 2007). All organisms are incubated at light except *Synechococcus elongatus* PCC 7942. Cells are concentrated when incubated with DNA in most protocols. Incubation in medium, water and buffer, and prior washing steps are protocol dependent as well as the time required for expression of the selection marker. Growth phase dependency of natural competence was shown in most cases.

One point of optimization for the NT protocol of *Phormidium lacuna* HE10DO derived from the other protocols was not discussed so far: Separation of time for NT and subsequent cultivation to express the selection marker. This could be considered for further protocol optimization. DNA and concentrated cells should be incubated for various time spans under standard cultivation conditions before they are transferred to cultivation medium. An additional benefit would be that conditions during DNA uptake could be varied for optimization of NT independently from cultivation conditions (as illumination, pH, salt and nutrient composition, and temperature for instance). Since *Phormidium lacuna* tolerates kanamycin quite good for some time, probably no time for selection marker expression is required and cells could directly be transferred on selective medium after NT.

In general, the different NT protocols in literature show which of the parameters can be crucial and indicate a range in which NT was already demonstrated. All parameters mentioned in the protocols of other NCC are in accordance with the discussion above. Homogenization was not part of any published NT protocol but may be promising to be tested in future since this may be a crucial part in NT of *Phormidium lacuna*. Sonication and EP were tested for *Synechocystis* sp. PCC 6803 but it did not show beneficial effect (Zang *et al.*, 2007). Anyhow, certain pretreatment may be supportive for other cyanobacteria. For mechanical and ultrasonic homogenization as well as EP, organism specific

parameters have to be tested. For filamentous cyanobacteria homogenization in transformation protocol is common, like for *Nostoc punctiforme* PCC 73102 with a sonication step in an EP protocol (Holmqvist *et al.*, 2009).

The elimination of EPS and nucleases to a certain level (by homogenization and subsequent centrifugation) is one explanation model for NT in *Phormidium lacuna*. If homogenization is critical, this would explain why NT was not found for several other cyanobacteria. But also common factors of the transformation protocols of other NCC were considered from the beginning on (cell concentration, washing) or during the restoration (conditions during DNA uptake) and optimization of transformation (growth phase). The combination of these conditions with addition of homogenization most likely enabled NT in *Phormidium lacuna*. All factors have to be considered when testing for NT among CNT^+, including moderate mechanic stress, which would be the input exclusively derived from this work. The most suitable organism to characterize all the factors that might have impact on NT are the other strains of *Phormidium lacuna* GI09CO and HE10JO, since both of them were already transformed with the EP protocol I. Thus, it should also be possible to restore transformability for them based on all experiences gathered for HE10DO and the relevant factors discussed above.

4.3 Further development of molecular biology in *Phormidium lacuna*

In the previous section several optimization approaches for improved NT protocols are discussed. Most of them deal with the question how to further characterize and optimize NT, also in respect of the potential transferability to other cyanobacteria. In the following, aspects are discussed that address the potential application of *Phormidium lacuna* in molecular biology and biotechnology.

The optimization of the NT protocol is also important in this respect. The reduced transformation efficiencies for plasmids encoding for the enzymes PDC or PDC/ADH (Figure 13) show that longer inserts between the flanking, homologous sequences reduces recombination frequency. This is in accordance with literature for other bacteria (Kung *et al.*, 2013). Therefore, the points described for NT protocol optimization above are also relevant for further biotechnology application.

The characterization of *Phormidium lacuna* for biotechnology via heterologous ethanol production did not show conclusive results (3.3). The reasons why no elevated ethanol levels could be detected for the relevant clones is discussed under 4.4. One possible reason is a weak promoter activity. Thus, promoter assays are a required point for the further development, not only for ethanol production but heterologous expression in general. Presently, experiments for the establishment of luciferase assays are performed by students of our workgroup in their bachelor or master thesis (Ferdinand Betting, Finn Zaiß), but respective transformants are not available until now. Also a fluorescent protein like GFP (green fluorescent protein) as expression marker would be possible. An approach with a GFP encoding plasmid (7-GFP_Kn) in this work did not result in transformants (electronic supplement: transformation experiments). The expression of GFP in 7-GFP_Kn is under control of the *cpcB* promoter of *Synechocystis* sp. PCC 6803, a promoter for which high expression rates were reported in

Synechocystis sp. PCC 6803 (Zhou *et al.*, 2014). Further GFP plasmid designs also with endogenous promoters of *Phormidium lacuna* like the *psbA* promoter (Photosystem II protein D1) were considered, but they were not completed due to complications during the cloning process. The existing GFP plasmid can be used for further work, for instances by transferring the approach of longer homologous sequences also on this design. Fluorescent proteins have the advantage that they can be detected in the living cell and it may be possible to identify transformed cells on agar plates very early. It would probably also possible to calculate transformation rates more precisely. However, as a photosynthetic organism *Phormidium lacuna* shows considerably autofluorescence and lower expression level of a fluorescent marker may be hard to detect. Monitoring promoter strength via luciferase expression does not have this limitation but it cannot be performed in vivo. Once a successful system is established to monitor promoter strength, promising endogenous promoters could be tested as well as promoters that show good expression rates in other cyanobacteria. One possible example would be the P_{cpc560} promoter, a super-strong promoter described for *Synechocystis* sp. PCC 6803 (Zhou *et al.*, 2014). The identification of promising promoters is probably the most central part in the further development of *Phormidium lacuna* as interesting organism for biotechnology and as potential model organism. After establishment of the NT transformation protocol, promoter assays and heterologous ethanol production are the two most important steps to continue. Both steps were performed in parallel in our workgroup since the high kanamycin resistance of transformants was a possible indicator of considerably high promoter strength. But this is probably not the case. Steps towards ethanol production were performed in this work, while establishment of promoter assays were students projects (see above). Since respective plasmids and transformants for ethanol production are available by this work already, probably only respective promoters have to be exchanged and tested for performance. Because transformation efficiency probably decreases with increasing insert size, it should be considered to exchange the promoter via homologous recombination into the existing transformants and not on the plasmid level. The use of a new selection marker would be required therefore.

The use of new selection markers, namely other antibiotic resistances, would be helpful for further modifying existing clones. It would also be possible to investigate if the high resistance of transformants is only true for kanamycin or also other antibiotics. Since transformation efficiency probably decreases with insert size, the introduction of more complex metabolic pathways requires more than one integration site. For every integration site a new selection marker would be required. Presently, the introduction of riboflavin synthesis into HE10DO is one project of our workgroup in cooperation with the Institute of Technical Microbiology (Mannheim University of Applied Sciences, Germany).

As mentioned, different integration sites have to be tested. In this study only the locus 07_37 was tested positively, while two other loci did not work for transformation. Notably for respective plasmid 7-K, the *kanR* operon is on both sides flanked with longer sequences than 2-K and 55-K. Thus, it may

be related with the longer homologous sequences that the locus 07_37 worked for transformation but the other two did not. However, the plasmids 2-K and 55-K were only tested in two evaluable experiments and thus it cannot be stated that the respective loci are not suitable for transformation in general. Since the integration in the respective mutants is always connected with the interruption of an ORF, it would be generally be possible that transformation does not work because the respective mutation is lethal. For the plasmids 2-K and 55-K this is highly unlikely, because the annotated function (see 3.1.1). In general, the integration into the genome should be possible at nearly every position at first because of the polyploid nature of *Phormidium lacuna*. However, for the knockout of essential genes it would not be possible to select homozygous mutants. New loci as integration sites are necessary for further work with transformants and for the integration of complex pathways. Also certain knock-out mutants could be interesting (see also 4.4). Respective plasmids were generated by Vera Aschmann (bachelor thesis) and the generation of transformants is currently under investigation by Finn Zaiß (master thesis). As a consequence of this work and in concordance with literature (Zang *et al.*, 2007), 1000 bp homologous sequence length are used on both sides of the *kanR* insert.

Another possibility to increase expression of recombinant proteins is to increase the gene dosage, which was already demonstrated for metabolic engineering in cyanobacteria (Dexter *et al.*, 2015). The integration in multiple loci on the genome (see above) and the use of self-replicating plasmids are strategies to achieve higher gene dosage. So far, there is no evidence for native plasmids in *Phormidium lacuna*. For other cyanobacterial species self-replicating plasmids are described that might be also usable in *Phormidium lacuna*. For example, a self-replicating shuttle vector was designed based on a native plasmid of *Synechococcus elongatus* PCC 7942 (ten plasmid copies per chromosome), which can also replicate in *Anabaena* (Chen *et al.*, 2016). In this work the use of the self-replicating plasmid pVZ321 did not result into transformants. But this was only tried in time periods of low transformability or low cell viability with EP protocols. Thus, the transformation of self-replicating plasmids could be tried again with the NT protocol.

As discussed above, nucleases can interfere with the transformation efficiency. The influence of nucleases on plasmid DNA during the transformation protocol was not tested in this work, but would be a further step in optimization. While the removal or inhibition of nucleases and protection through methylation of restriction sites were already discussed in 4.2.2, one could also consider making knockout mutants of the respective nucleases. In *Thermosynechococcus elongatus* BP-1, the disruption of a nuclease gene increased transformation rates and also allowed the transformation with so far not transformable plasmids (Iwai *et al.*, 2004). Also for *Synechocystis* sp. PCC 6803, the deletion of the exonuclease RecJ increased the transformation rates drastically (Kufryk & Vermaas, 2001). Possible candidates for deletion are restriction enzymes (Table 17) and further desoxyribonucleases. For identification the genome sequence data of HE10JO can be considered (Nies *et al.*, 2017).

The use of the CRISPR/Cas system (CRISPR: Clustered regularly interspaced short palindromic repeats, Cas: CRISPR associated) in cyanobacteria and its benefits is summarized in reviews (Behler

et al., 2018, Naduthodi *et al.*, 2018). With the ability to generate site specific double strand breaks, deletion mutants can be generated and the rate of homologous recombination can be increased. Last could be especially interesting when transforming plasmids with big inserts. Deletion mutants can be useful to eliminate nucleases or for the subsequent removal of selection markers. In a biotechnology oriented approach, like ethanol production, the carbon flux towards the desired product can be increased with the deletion of competing pathways like phl#2712 (see 4.4) without the need of the integration of a selection marker at the respective site. CRISPR/Cas mediated increase of homologous recombination can positively affect transformation efficiency as long as this is a rate limiting step (for example concerning plasmids that are hard to transform). Even though the CRISPR/Cas system brings many advantages and possibilities in genetic engineering, its application in *Phormidium lacuna* should be rather a long term goal. More basic points discussed above like new insertion sites, promoter assays, and general optimization of the transformation protocol are more relevant for the nearer future.

4.4 Ethanol production

Ethanol levels produced by HE10DO transformants did not differ from the ones of the WT, thus no heterologous ethanol production could be detected (Figure 19). Native production of ethanol and other fermentative products has been reported for other cyanobacteria (Heyer & Krumbein, 1991). Insertion of the relevant sequences in the genome was like intended. This was proved by sequencing of PCR products covering the whole integrated sequences. Neither for the *Synechocystis* sp. PCC 6803 or *Phormidium lacuna* HE10DO transformants encoding for pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) elevated ethanol production could be monitored nor for the HE10DO transformants encoding PDC alone. Overexpression of PDC only showed ethanol production for transformants of *Synechocystis* sp. PCC 6803 (Dexter *et al.*, 2015), possibly due to expression of endogenous ADH.

For *Synechocystis* sp. PCC 6803 transformant no elevated ethanol production could be detected in comparison to the respective WT or *Phormidium lacuna* cultures. In fact, the absolute ethanol level in the supernatant was the highest for *Synechocystis* sp. PCC 6803 transformant petJ PA, but in relation to cell density all samples were in the same range of ethanol concentration. *Synechocystis* sp. PCC 6803 petJ PA was generated earlier in our workgroup and tested several times positive for ethanol production in student's courses with photometric assays. The principle of the used assay is the same described under 2.4.1 but the protocols differ in details. The former results for ethanol production of *Synechocystis* sp. PCC 6803 petJ PA are contradictory: Sometimes ethanol production could be monitored and sometimes not. In this study clearly no ethanol production could be monitored. Since gas chromatography (GC) measures ethanol directly, while in the photometric assay the production of NADH is measured, the GC data is considered more relevant. A change in NADH level can have several reasons, turnover of other substrates through ADH due to low substrate specificity (Nealon *et al.*, 2015) or the presence of other enzymes that catalyzes the reduction of NAD to NADH. This is

especially relevant since in the respective protocol of the student's course culture supernatant but also cell lysate are used for analysis. A phenomenon in this work during photometrical ethanol assay (Table 6 in 2.4.1) was that false positive results could be monitored due to precipitation of medium or cell culture components. If the ratio of cell supernatant to reaction solution was too high, an unidentified precipitate was built and an unspecific rise of OD_{340} was monitored. This might be an explanation for false positive results during student's courses, since both protocols are related. Furthermore, the respective transformants *Synechocystis* sp. PCC 6803 petJ PA was generated with a plasmid that still has the promoter of *Zymomonas mobilis* between the endogenous promoter *petJ* and the enzymes PDC and ADH. It is possible that the promoter of *Zymomonas mobilis* enables no or only week gene expression in *Synechocystis* sp. PCC 6803 and the efficiency of the *petJ* promoter is impaired by the additional sequence in between. Furthermore the *petJ* promoter is induced by Cu^{2+} depletion. With Cu^{2+} only low expression is possible (Kuchmina *et al.*, 2012). Cu^{2+} should be removed by addition of bathocuproine, which build complexes with Cu^{2+}. However, bathocuproine is hardly soluble in water and most bathocuproine in aqueous solution precipitate. Bathocuproine was used in the former approaches and was also transferred to this work. In future, copper depletion medium would be a more promising approach for future analysis in contrast to bathocuproine.

While missing ethanol production can be explained for *Synechocystis* sp. PCC 6803 with its genotype and missing copper depletion, other explanations are relevant for HE10DO transformants. For the transformants 7-P-K:2k and 7-P-A-K:2k the expression of the respective proteins can also be considered as given since these clones also show a resistance to kanamycin. The expression of all proteins is enabled by the same promoter. Thus, also the genes upstream of *kanR* should be transcribed into mRNA. This is not the case for the transformants *Phormidium lacuna* HE10DO 7-pP_K:2k and *Synechocystis* sp. PCC 6803 petJ PA, where the selection marker is under control of a separate promoter. In terms of translation, the codon usage of the *pdc* and *adh* gene might be different to the *kanR* gene. Since the expression of PDC and ADH with *Zymomonas mobilis* origin work in other cyanobacteria, this is not regarded critical.

The expression strength of the *kanR* promoter was first considered high, because of the high resistance of *Phormidium lacuna* transformants towards kanamycin. Considerably high antibiotic resistance can be reasonably explained with high expression of the selection marker, but also other reasons are possible (see 4.1.2). A low activity of the *kanR* promoter would be a possible reason for the missing heterologous ethanol production in the respective *Phormidium lacuna* transformants. So far, resistance towards kanamycin is the only reference for the *kanR* promoter strength in *Phormidium lacuna*. The attempts by other work group members to monitor strength of the promoter on the mRNA level, to generate new transformants for luciferase assays, or to detect the enzymatic activity of the *kanR* gene product did not work so far. Also an initial attempt to use GFP as reporter system in this work did not result in transformants. In future, these attempts should be intensified since a reliable system to

monitor promoter strength is a mandatory feature to further classify *Phormidium lacuna*'s potential for biotechnology (see 4.3).

Low or no activity of PDC or ADH would be an additional explanation why no ethanol could be detected. The availability of cofactors (thiamine pyrophosphate for PDC, NADH for ADH) or a differing intracellular pH level from the enzymes optimum might cause limitations. There are several ways to optimize the production of ethanol by PDC and ADH in cyanobacteria (see 1.1), which could also be tested in *Phormidium lacuna*. But first the sufficient expression of PDC and ADH should be achieved by the use of characterized promoters and be confirmed by western blots.

A further point to consider is the consumption of ethanol or its precursor acetaldehyde through competing pathways. In fact, there is an endogenous pathway in *Phormidium lacuna* to produce ethanol out of acetyl-CoA (Nies *et al.*, 2017). This enzyme or other endogenous ADHs explain the ethanol production for HE10DO WT determined by GC. Because there are no significant differences to all HE10DO transformants, either this pathway overlays the recombinant pathway or there is no heterologous ethanol production at all. The relevant protein for the reaction of acetyl-CoA or acetate to ethanol is a bifunctional acetaldehyde dehydrogenase/alcohol dehydrogenase, phl#2712 (Nies *et al.*, 2017). This reaction is reversible in contrast to the heterologous way, in which the decarboxylation of pyruvate is irreversible (Figure 2 in 1.1). This means that the endogenous pathway can also consume ethanol or acetaldehyde and can oxidize it to acetate or acetyl-CoA. This would be a third possibility for no detectable heterologous ethanol production. The role of bifunctional acetaldehyde dehydrogenase/alcohol dehydrogenase in photoautotroph organisms is not completely understood. The enzyme plays a key role in the anaerobic redox balance in fermenting bacteria and may play a role in stress response and low light conditions in cyanobacteria (van Lis *et al.*, 2017). Since this pathway is reversible, it could also lead to the consumption of ethanol in HE10DO transformants. Notably, the protein phl#2712 seems to be absent in *Synechocystis* sp. PCC 6803, *Synechococcus elongatus* PCC 7942, and *Synechococcus* sp. PCC 7002. These three organisms are also the organisms that are most frequently used for the heterologous production of ethanol. The protein sequence of phl#2712 was compared with protein sequences of the order Synechococcales and the cyanobacteria in total with the online version of BLASTp (default settings, non-redundant protein sequences database, 10.12.2018). Homologs with the same annotation are found for various cyanobacteria, also in the order Synechococcales, but not for the model organisms mentioned above. Since this protein is not present in the strains frequently used for recombinant ethanol production, there is no experimental data in literature to compare with the potential influence of phl#2712 in the respective transformants of *Phormidium lacuna*. The coding sequence of this protein would be an ideal candidate when testing for new integration sites in the genome (see 4.3). It would be interesting to characterize recombinant ethanol production in phl#2712 knockout mutants and derived transformants for ethanol production.

Even though the qualitative comparison of the ethanol content of the different samples is considered relevant, the GC measurement has some limitations in this work. For further research on ethanol

production not only new transformants but also a refinement of the current method is required. Possible approaches are the use of a fresh column to reduce background, automatic sample injection to reduce variations caused by the experimenter, and splitless injection to increase amount of ethanol on the column. Since ethanol is a volatile compound and the salt rich, aqueous medium might not be optimal for a wax column, also head space GC could be considered. For the present transformants either no or only small ethanol production would be observable that could not be monitored sufficiently with the present precision of the GC method. Concerning the samples supplemented with glucose, the relative ethanol concentration was lower for WT and 7-Kn:2k in comparison to the standard medium in this case. In contrast, it was at the same level for 7-P-A-K:2k. If ethanol is produced by the respective transformants, it is at such low levels that the differences are below threshold and are more precise measurement would be necessary. *Phormidium lacuna* might be capable of mixotrophic growth (Nies *et al.*, 2017). Additional glucose in the medium might increase the intracellular pyruvate level and thus the ethanol production.

The possible reasons above for the undetectable heterologous ethanol productions underline the necessity for a system to monitor promoter strength. Especially since at least for two transformants the expression of PDC alone or PDC and ADH together is most likely due to their kanamycin resistance (P-K:2k and 7-P-A-K:2k). Promoter assays would clarify if promoter activity is the crucial point in the existing transformants. Also deletion mutants of phl#2712 in WT and P-K:2k and 7-P-A-K:2k transformants would probably be a critical point in enabling ethanol production in *Phormidium lacuna*. Thus, the recombinant production of ethanol or other compounds in *Phormidium lacuna* relies strongly on the further development of molecular tool box (4.3).

Heterologous ethanol production is on one hand an indicator for the suitability of *Phormidium lacuna* transformants in biotechnology and on the other hand can be considered as a sustainable way to produce biofuels (see also introduction). Once ethanol production is achieved, there are several ways to optimize ethanol production that have been reviewed in cyanobacteria (see also 1.1). Since there are several reasons why ethanol production was undetectable in HE10DO (mentioned above), the heterologous production of other compounds could be tested, preferably lactate as alternative model compound. This reaction is only based on one reaction step, was already demonstrated in cyanobacteria, and lactate can be used as precursor for bioplastics (Niederholtmeyer *et al.*, 2010, Angermayr *et al.*, 2012). Reliable promoters however are also required for this approach. The general ability of *Phormidium lacuna* to grow in bioreactors has already been demonstrated (Nies *et al.*, 2017). At the moment, further experiments are performed by Nora Weber (master thesis) to optimize the cultivation in bioreactor and to include the filamentous and adherent growth properties of *Phormidium lacuna*.

4.5 Prediction of candidates to test for natural transformation

Four orders of cyanobacteria comprise examples for NT: Few genera are known for the Synechococcales, and one species each for Chroococcales, Nostocales and Oscillatoriales. These four, taxonomically wide spread orders of cyanobacteria are also the one with the majority of sequenced genomes (> 90%, NCBI/genomes[5]) and on which research focuses. The examples of NT within four major orders of the cyanobacteria is more likely to be explained by a much broader distribution of NT among cyanobacteria than by several independent and exceptional findings.

If NT is wider distributed among cyanobacteria, many species would be easily accessible for genetic manipulation. This would increase the potential of applied and basic research in the cyanobacterial field. A broader distribution of natural competence might also imply that there is a conserved and important function for the cyanobacteria, which has to be investigated (see also 4.2).

A first analysis based on the homology of NTFs between six NCC and a representative selection of cyanobacteria showed that a broad range of organisms could be identified as CNT^+. NT was often considered to be an exclusive trait of unicellular cyanobacteria. In contrast, in Table 16 all CNT^- (non-promising candidates to test for natural transformation), except one, are unicellular cyanobacteria. The selected cyanobacteria in this study are just a small representation of all available cyanobacterial genomes, but this could imply that NT is more likely to be found among filamentous cyanobacteria with the right method. Perhaps research focus on easily cultivable and naturally transformable unicellular cyanobacteria of the genus *Synechocystis* and *Synechococcus* led to the misapprehension that NT can be exclusively found for a small and distinct group of unicellular cyanobacteria. The fact, that NT was not found for any filamentous cyanobacterium with one expectation in the early 80s (Trehan & Sinha, 1982), the same that year natural competence was first described for *Synechocystis* sp. PCC 6803 (Grigorieva & Shestakov, 1982), is a reasonable support for this assumption. NT in *Phormidium lacuna* was only demonstrated with a certain pretreatment so far that is discussed to be presumably essential: At least homogenization and one centrifugation step. Since homogenization is not included in any NT protocol (see 4.2), this might be a reason why NT was potentially overseen for other filamentous cyanobacteria.

To make further predictions about the distribution of natural competence among cyanobacteria more genomes have to be analyzed with the methods used in this study. Depending on the number of monitored genomes the development towards a somehow automated process would be advisable: The analysis in this study was performed completely by hand (36 genomes), but the number of sequenced cyanobacterial genomes exceeds this number considerably (501 genomes, NCBI database, 27.11.2018).

By now 23 cyanobacteria were predicted as CNT^+. It would be reasonable to test first in closely related cyanobacteria of the genus *Phormidium* and *Microcystis* if there is a potential for NT in further cyanobacteria. Also the transfer of the developed NT protocol on other *Phormidium lacuna* strains

[5] https://www.ncbi.nlm.nih.gov/genomes/GenomesGroup.cgi?taxid=1117 (27.11.2018)

would be advisable, before testing more distantly related organism. The possible experimental steps to consider are discussed in the sections above (see 4.2). Also it could be an interesting option to test promising candidates for type IV pilus mediated motility since functional type IV pili are considered essential for motility and NT as well. In fact, gliding motility in filamentous cyanobacteria is poorly understood even though latest result implies a functional role of the type IV pili in the order Nostocales (Khayatan *et al.*, 2015, Cho *et al.*, 2017). The capability of *Phormidium lacuna* for gliding motility and NT alike, while last requires functional type IV pili, implies that also gliding motility in the order Oscillatoriales might be linked to type IV pili. However, experimental proof by respective knock-out mutants would be required.

For some of the CNT^+ transformation protocols already exist. For example, there are EP protocols for *Cyanothece* sp. ATCC 51142 (Min & Sherman, 2010) and *Nostoc punctiforme* PCC 73102 (Holmqvist *et al.*, 2009) as well as for other strains of *Arthrospira platensis* (Toyomizu *et al.*, 2001) and *Leptolyngbya boryana* (Fujita *et al.*, 1992). Since transformability was already demonstrated and NT cannot be excluded in most EP protocol (like already discussed), these CNT^+ would possibly be good organisms to screen for NT. Especially because *Cyanothece* sp. ATCC 51142 and *Arthrospira platensis* NIES-39 are among the most promising CNT^+. For instance *Cyanothece* sp. ATCC 51142 possesses the highest values for the sum of the highest minimum homology quotient (Table 15) and the mean of the average homology deviation (Table 16) beside the close *Phormidium* and *Microcystis* relatives of the respective NCC. Thus, based on the results in 3.4 *Cyanothece* sp. ATCC 51142 is the most promising candidate to test for NT in a new cyanobacterial genus.

Interestingly, *Phormidium lacuna* shows high homology only with some *Phormidium* species, while the homology to other members of the genus *Phormidium* is comparable with the overall homology in the order Oscillatoriales. This is presumably explainable with the polyphyly of this genus and indicates that the taxonomic classification of the genus *Phormidium* is not final (see also 1.2.4).

Finally, it should be mentioned that this whole analysis relies on a homology based prediction. Already one single point mutation can lead to a loss of function of essential NTF. Thus, the respective cyanobacterium would not be naturally transformable anymore. It is hypothesized that the ability of NT is common in the domain of bacteria, but that it is frequently lost on different taxonomic levels (Mell & Redfield, 2014). This would explain why for some examples only one missing NTF was found, although this missing might be explained also with incomplete genome data in single cases. Additionally, only genomic data are considered in this approach. In case a complete set of NTFs is encoded on the genome, this does not necessarily mean that they are all expressed and functional at the same time. Thus, also a promising classified cyanobacterium can completely miss NT. Also growth phase dependency of NT and the influence of other factors are necessary to consider and are discussed in the sections above.

It should be mentioned that the prediction of NT relies on the finding in six NCC. Since the conservation on the NTFs is not that high, further distantly related species may not be predicted by a

homology based approach. Furthermore, the overall low homology level homology level among the NCC includes most of the analyzed cyanobacteria and predict them as CNT^+. Findings that would not support this hypothesis would have been an overall high homology of the NTFs among the NCC and distinct lower homology towards the NTF of all other cyanobacteria. Or the clear missing of at least one NTF in all analyzed cyanobacteria except the NCC. But this is not the case.

The overall low homology among the NCC can already be seen with *Synechococcus elongatus* PCC 7942. This species has, without self-alignment, lower values for the mean of the average homology deviation (Table 16) than any of the CNT^+. However, this is also the organism, for which NT was described first in cyanobacteria (Shestakov & Khyen, 1970) and which is used frequently as model organism today. This illustrates that high homology is not a critical criterion to be a promising candidate as long as all NTFs can be found in the genome. In contrast, the overall low homology among the NCC, best illustrated by *Synechococcus elongatus* PCC 7942, is a central point that allowed the comparison of organisms among the whole phylum of cyanobacteria. The comparison with just one cyanobacterium like *Synechocystis* sp. PCC 6803 would not have resulted in such consolidated finding. The comparable, overall medium homology concerning the NTF among the NCC and the majority of the other cyanobacteria implies furthermore that NT might be a common trait among many cyanobacteria. Thus, all cyanobacteria classified as CNT^+ are worthy to test for NT.

5 Conclusion of this work

Natural transformation (NT) would be the preferable transformation method in cyanobacteria, at least as long as efficiency in the respective organism is reasonable high. Methodical requirements are low and pretreatment is gentle. Also single recombination events, which are often undesired, are most likely to take not place during NT.

With the discovery of NT and the establishment of a reliable transformation protocol, numerous branch points for subsequent work and following projects arose. The establishment of transformation is a crucial point to make *Phormidium lacuna* accessible for molecular biology and in consequence several basic and applied research projects are possible. The further optimization of the transformation protocol and the development of molecular tools for *Phormidium lacuna* have huge impact on the chances for success of future projects and is thus obligatory. Since transformation of *Phormidium lacuna* works already very reliable and there is still much potential in further optimization, the strain HE10DO may be suitable as model organism for filamentous cyanobacteria without heterocysts. An additional reason for this is the fast segregation of heterologous sequences in transformants. Interesting research objectives would be nitrogen fixation, of which *Phormidium lacuna* is capable, the mechanisms underlying the ability for gliding motility, and the finding of NT itself.

In an applied aspect the heterologous production of valuable compounds like ethanol will benefit from improved genetic accessibility. Further research is required that is predominantly linked to the further development of the molecular toolbox for *Phormidium lacuna*. The characterization of cultivation properties in bioreactors is additionally reasonable for *Phormidium lacuna* as a genetic manipulable organism. Especially the utilization of *Phormidium lacuna*'s abilities to grow on surfaces and to build aggregates during bioreactor cultivation is probably a promising point for further research.

The unforeseen finding of NT has many implications beyond optimization of transformation procedures for *Phormidium lacuna* and may also be important for cyanobacterial research in general. Since NT is found in an additional order of cyanobacteria, it implies that NT is a much more common trait in this phylum that was largely overseen until now. Bioinformatics analysis underlines this probability and promising candidates to test this hypothesis could be identified. The consequence of much more cyanobacteria being easily genetically accessible may have significant implications for further basic and applied research with cyanobacteria.

6 Supplement

The supplement of this work comprises the figures and the text below. Additionally, electronic files are attached to this work. The following files can be found in the electronic supplement: “bioinformatics”, “plasmids – maps and sequences”, and “transformation experiments”.

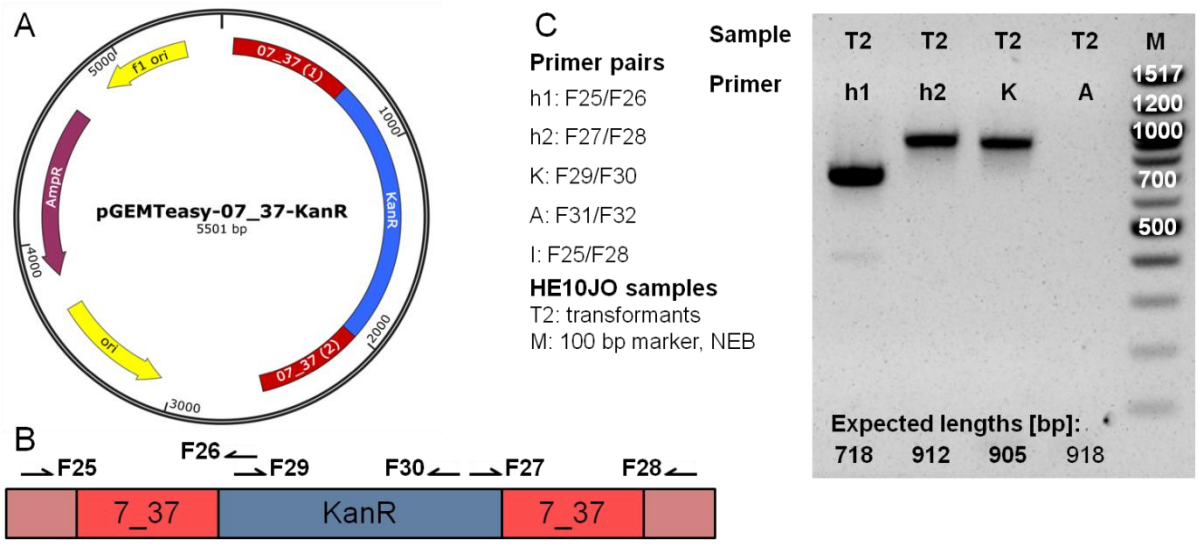


Figure 20: Validation of transformation, additional PCR results. A) 7-K plasmid (full name: pGEMTeasy-07_37-KanR): red - homologous sequences of the 07_37 locus, blue - kanamycin resistance operon, yellow - origin of replication (f1 - bacteriophage origin, other - pUC origin for *Escherichia coli*), purple - ampicillin resistance operon. **B) Integration site of the 7-K plasmid with primer binding sites.** red - homologous sequences encoded on the vector, pale red - *Phormidium lacuna* chromosome, blue - kanamycin resistance gene. **C) Validation of transformants - test for different sequences with PCR.** Agarose gel for the reaction with primer pairs that cover different parts of *Phormidium lacuna* transformants or the plasmid used for transformation. Primer binding sites indicated in B. Primer pair A binds on *ampR* operon. M: 100 bp DNA ladder (NEB, USA). T2: Sample from the second transformation experiment.

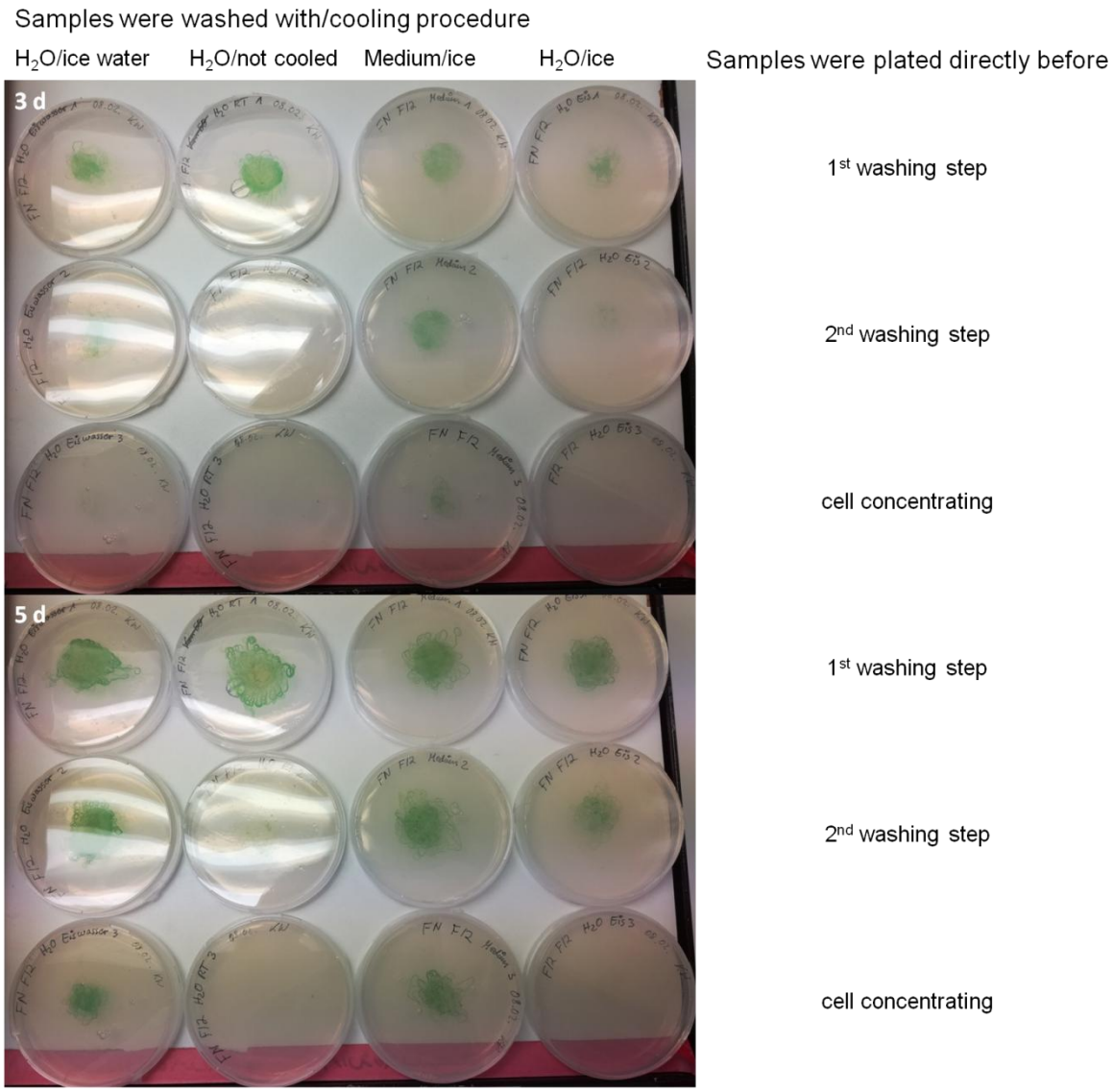


Figure 21: Propagation of *Phormidium lacuna* samples on agar plates at two time points in dependency of washing medium and cooling. Samples were plated at the first and the second washing step with water, and after cell were concentrated for electroporation. Samples were cooled either with ice or ice water or samples were not cooled at all. In one case, cells were not washed with water but with f/2 medium.

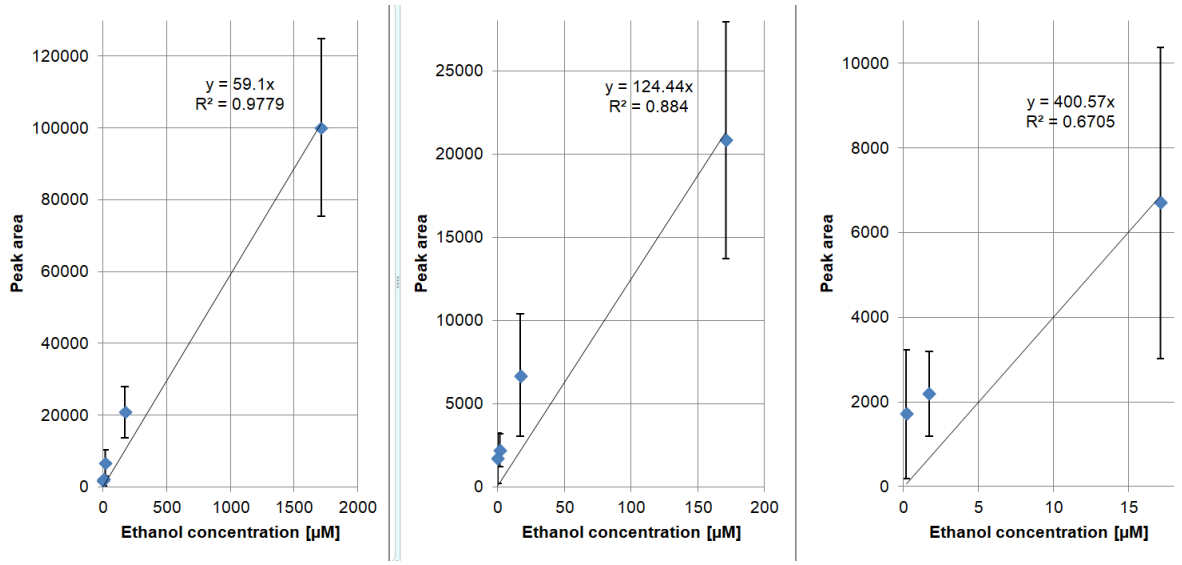


Figure 22: Ethanol standard curves. Peak area is set in relation to ethanol concentration [µM]. Depending how many concentration steps are monitored, the slope of the straight varies. Left equation was used to calculate ethanol concentration.

Ethanol standard curve: The measured samples for ethanol standard curve were dilution in 10^{-1} steps, thus differences between the different peak areas in the basis of ten were expected. The straight's slope does not fit to this. Depending on how many data points are used the straight's slope also varies drastically. However, this is the best possibility available to estimate the ethanol concentration of the culture's supernatant in the respective measurements. The left equation was used for the ethanol calculation, since it has the most data points. Possible explanations for these variations are a non linear relation between amount of ethanol and FID signal or variations in the injection that resulted in different amount on ethanol on the GC column. Background from the column interfering with the measurement is only relevant for the lowest concentration tested for the ethanol standard curve.

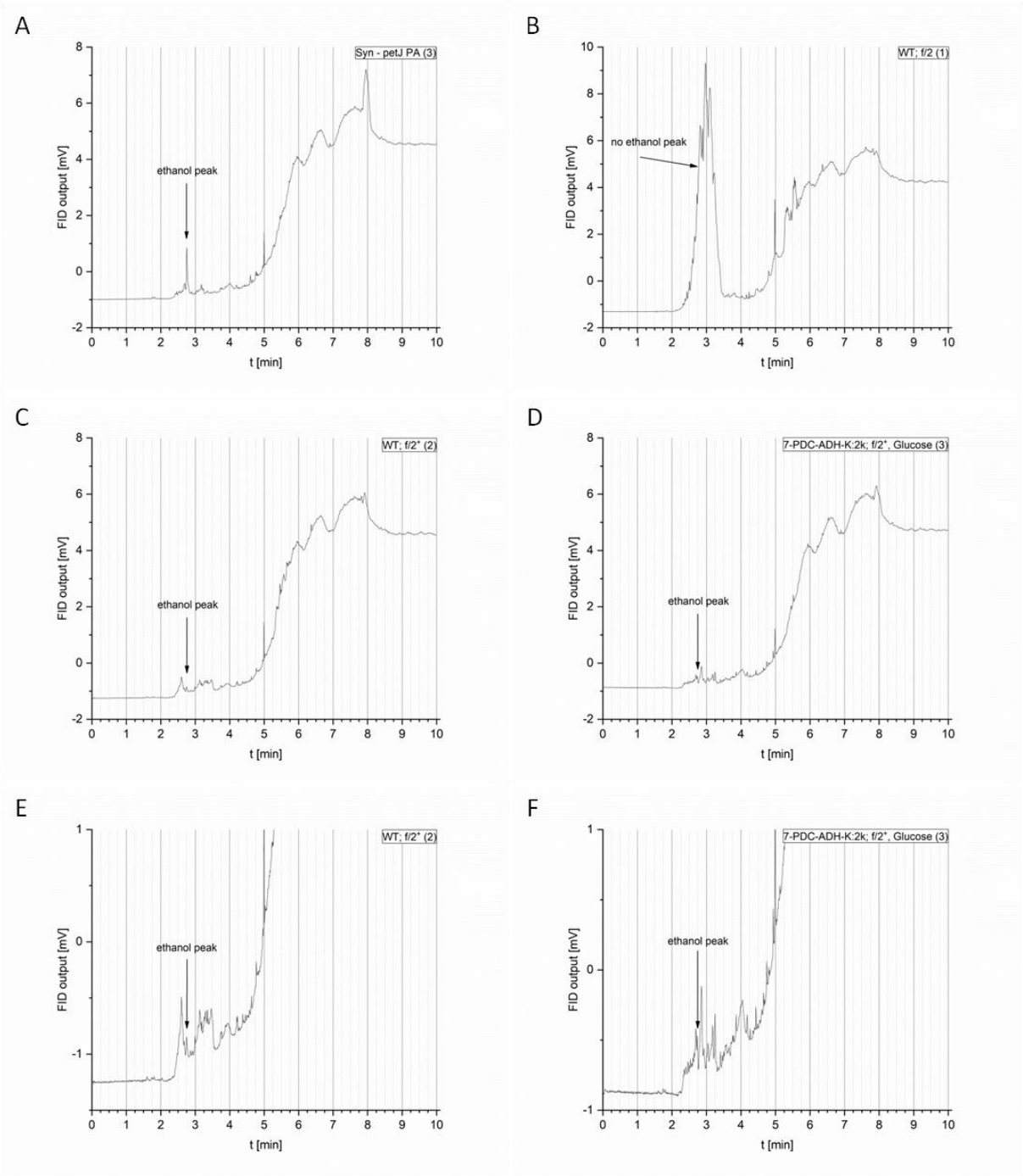


Figure 23: Examples of gas chromatography primary data. The FID output [mV] is blotted against the running time of the program [min]. The ethanol peak is indicated if possible (retention time: 2.75 s ± 0.01 s). Sample and number of experiment (brackets) are indicated. A: comparative strong peak, reliable area calculation by integration is possible. B: High background, no ethanol detection possible. C+D: weak peak, reliable area calculation is still possible because low background. E+F: ethanol peak detectable, reliable area calculation hardly possible due to high background. FID: flame ionization detector.

Table 18: Accession numbers (GenBank or RefSeq) for the natural transformations factors of *Synechocystis* sp. PCC 6803 and their best hits in the other NCC. NCC: natural competent cyanobacteria.

Annotation	*Synechocystis* sp. PCC 6803	*Synechococcus elongatus* PCC 7942	*Synechococcus* sp. PCC 7002	*Thermosynechococcus elongatus* BP-1	*Phormidium lacuna* HE10JO	*Microcystis aeruginosa* PCC 7806
ComEA-family	BAA10416.1	WP_011377526.1	WP_012307508.1	NP_683129.1	WP_087705189.1	CAO90699.1
ComEC-family	BAA17126.1	WP_011378473.1	WP_012306455.1	NP_682492.1	WP_087708507.1	CAO86447.1
ComF-family	BAA10110.1	WP_011378447.1	WP_012305695.1	NP_682126.1	WP_087710481.1	CAO87311.1
DprA	BAA17376.1	WP_011377689.1	WP_012308102.1	NP_682221.1	WP_087705442.1	CAO87006.1
PilA1	BAK49923.1	WP_011377418.1	WP_012308390.1	NP_681801.1	WP_087711780.1	CAO87025.1
PilB1	BAK51077.1	WP_011378301.1	WP_012306325.1	NP_680913.1	WP_087705667.1	AAY51450.1
PilD	BAK48812.1	WP_011244469.1	WP_012308199.1	NP_681266.1	WP_087707065.1	CAO87378.1
PilM	BAK50448.1	WP_011243962.1	WP_012306132.1	NP_683131.1	WP_068788809.1	CAO86670.1
PilN	BAA18276.1	WP_081429509.1	WP_012306133.1	NP_683132.1	WP_087705201.1	CAO88032.1
PilO	BAA18277.1	WP_011378469.1	WP_012306134.1	NP_683133.1	WP_087705200.1	CAO88032.1
PilQ	BAK50451.1	WP_011378468.1	WP_012306135.1	NP_683134.1	WP_087705199.1	CAO88035.1
PilT	BAK50741.1	WP_011378300.1	WP_012306326.1	NP_680912.1	WP_087705668.1	CAO86288.1
RecA	BAK51899.1	WP_011243477.1	WP_012306060.1	NP_682879.1	WP_087705299.1	CAO90208.1
SigF	BAK51747.1	WP_011244805.1	WP_012307534.1	NP_681527.1	WP_087706808.1	CAO90794.1

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Wissenschaftlich

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Nicht-Wissenschaftlich

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