Genetic tools for desiccation- and radiation-tolerant cyanobacteria of the genus *Chroococcidiopsis*

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Cyanobacteria living in arid environments represent an unexplored source of novel and/or particularly efficient molecules which underlie survival of high radiation, prolonged desiccation and extreme temperatures. Members of the genus *Chroococcidiopsis* are often the only photosynthetic prokaryotes in extremely dry deserts such as the Dry Valleys in Antarctica and the Atacama Desert in Chile. Furthermore they can cope with stressors not so far encountered in nature, e.g. high doses of UV and ionizing radiation. Genetic tools are available for desert strains of the *Chroococcidiopsis*, including gene transfer and gene inactivation. Plasmids maintained in these cyanobacteria have been developed which make it possible to monitor gene expression and in vivo localization of proteins. The use of these genetic tools in combination with the foreseen availability of genomic sequences will contribute to unravelling the molecular basis of *Chroococcidiopsis* desiccation and radiation tolerance as well as its biotechnological exploitation.

Keywords Cyanobacteria; *Chroococcidiopsis*; gene transfer; reporter genes

In memoriam of E. Imre Friedmann and Roseli Ocampo-Friedmann

1. Hot and cold desert strains of *Chroococcidiopsis*

Cyanobacteria are oxygenic phototrophic prokaryotes which appeared 3.5-2.5 billion years ago and were responsible for introducing oxygen into the atmosphere of primitive Earth. They now colonize every light-exposed niche in our planet, including several extreme environments [1].

In extreme hot and cold deserts where life has been pushed to its very limits such as the Dry Valleys in Antarctica or the hyper arid core of the Atacama Desert in Chile, the occurrence of members of the genus *Chroococcidiopsis* has been widely documented [2]. Depending on the geological conditions these cyanobacteria find refuge inside rocks by colonizing microscopic fissures (chasmoendoliths) or structural cavities (cryptoendoliths) of rocks, forming biofilms at the stone-soil interface under pebbles of desert pavements (hypoliths), or growing within halite deposits [3]. The discovery of endolithic microbial communities in the Dry Valleys in Antarctica [4] was remarkable, since the Dry Valleys had long been considered virtually sterile [5]. The hyper-arid core of the Atacama Desert is also considered the absolute dry limit for life on Earth and, along with the Dry Valleys, represents the closest equivalents of two Mars' two environmental extremes: cold and aridity [6]. In view of their ability to thrive in such extreme environments, strains of *Chroococcidiopsis* are being extensively studied in astrobiological research aimed at establishing the limits of life beyond Earth and at finding signatures of extant or past life on other planets, such as Mars [7].

How desert strains of *Chroococcidiopsis* can manage to survive extreme desiccation on Earth is still something of a mystery. It was recently speculated that their desiccation tolerance depends on the capability to avoid and/or repair otherwise lethal damage induce at every level of the cellular organization [8]. Such a capability also seems to guarantee their surviving stressors not currently encourented in nature, such as doses of ionizing radiation as high as 15 KGy [9] or exposure to a few minutes of a simulated, unattenuated Martian UV flux [10].

2. Genetic analysis

In the last thirty years great progress has been made in developing genetic systems for cyanobacteria. However since genetic manipulations appear feasible only for a few of them, only certain strains have been preferentially used as model systems to unravel the molecular basis of several aspects of their metabolism and developmental features [11-13].

Approaching the molecular biology of desert strains of *Chroococcidiopsis* immediately posed several difficulties. Their growth rate is relatively low, with a generation time as long 16 days, although some relatively fast-growing strains, with a generation time of a 4-5 days, are available [9]. Furthermore, cells accumulate thick, polysaccharide-rich envelopes, which impair an easy achievement of the cell lysis with a consequent low efficiency in the extraction of genomic DNA. Often heterotrophic bacteria grow within the external layer of cyanobacterial cell envelopes, frequently making such bacteria very difficult to remove.

2.1 Extraction of *Chroococcidiopsis* genomic DNA

The first step in addressing the molecular biology of such a puzzling cyanobacterium has been the development of a method to extract genomic DNA. The resistance of *Chroococcidiopsis* to lysozyme treatment for achieving cell lysis, was used to reduce the bacterial contamination. In fact, by using this treatment followed by an osmotic shock and incubation with DNase, the bacterial contamination was reduced three-fold; subsequently the cyanobacterial cell lysis was achieved by means of hot phenol and glass beads [14]. The extracted genomic DNA was employed as the template in polymerase chain reactions (PCR) and for the first time, a *Chroococcidiopsis* gene was identified by using degenerative primers designed based on conservative regions of the cell division protein FtsZ [14]. However, the extracted genomic DNA was resistant to several restriction endonucleases; this carried out the purification on cesium-chloride density gradient of the genomic DNA necessary for performing additional molecular assays such as Southern analysis [14].

A small scale genomic DNA purification protocol was also developed using reduced amounts of *Chroococcidiopsis* cells (10^6-10^8). After being resuspended with sterile distilled water, cells were subjected to three cycles of freeze-thawing, boiled for 5 min, and after centrifugation, the supernatant used as PCR template [8]. This protocol has been applied in producing genomic PCR fingerprinting based on primers derived from highly iterated palindromic sequences (HIP) and short tandemly repeated repetitive(STRR) ones, which are exclusive of cyanobacterial genomes [15, 16]. Virtually identical HIP1- and STRR-PCR fingerprints were obtained from dried, rewetted and liquid samples of *Chroococcidiopsis*, suggesting that HIP and STRR sequences are always kept accessible due to their role in promoting genome reorganization and DNA repair upon rewetting [8] as also reported for the desiccation-tolerant cyanobacterium *Nostoc commune* [17].

3. Gene transfer

It is well-known that the most important requirement for genetic manipulation is genetic transfer. Electroporation and conjugation are widely used to transform cyanobacteria, even though efficiency may vary depending on the restriction enzymes present in cyanobacteria [12, 13]. Compared to transformation, which is achieved by adding exogenous DNA and applying an electric filed, conjugation, which is mediated by cell-to-cell contact, is more efficient for transforming cyanobacteria. According to the triparental mating method [18, 19], a self-transmissible conjugative plasmid of the broad-host-range P-incompatibility-group, transfers itself from an *E. coli* mobilizer strain to a second *E. coli* donor strain which carries the non-conjugative, mobilizable plasmid (cargo) intended for transfer to the recipient cyanobacterium. The *E. coli* donor strain may also harbour a helper plasmid if methylation of the cargo plasmid is required. Mobilizable plasmids contain an origin of transfer (*oriT,* also known as *bom*, *b*asis *o*f *m*obilization) which is nicked by a mobilization protein (eventually provided in *trans* by the helper plasmid), and transferred as a single strand to the recipient cyanobacterium. Here, the fate of the incoming plasmid depends on the presence of a replicon allowing its replication or, alternatively, it must integrate into the cyanobacterial genome. It has been reported that broad-host-range IncQ group plasmids can replicate within representatives of the genus *Synechocystis* and *Synechococcus*, in *Anabaena* sp. PCC 7120, and *Prochlorococcus* sp. MIT9313 [11-13].

However the impossibility of IncQ-based plasmids to replicate inside other cyanobacteria has encouraged the search for indigenous plasmids. The aim is to construct shuttle plasmids carrying two replication origins capable for maintenance in *E. coli* and in the given cyanobacterium. Several cyanobacteria harbour one to many plasmids, which may range in size from 0.9-35.0 MDa [20]; among them plasmid, pDU1, from *Nostoc* sp. strain PCC 7524 has been used extensively in a variety of shuttle vectors [13].

3.1. Gene transfer to *Chroococcidiopsis*

In the effort to decipher the molecular mechanisms underlying the desiccation and radiation tolerance of desert strains of *Chroococcidiopsis*, the possibility to genetically manipulate them is extremely challenging. When the suitability of different strains to gene transfer was investigated, out of five strains, namely CCMEE 029 (Negev Desert, Israel), CCMEE 057 (Sinai Desert, Egypt), CCMEE 123 (coastal desert, Chile), CCMEE 171 (Ross Desert, Antarctica) and CCMEE 584 (Gobi Desert, Mongolia) only strains CCMEE 029, 057 and 123 were suitable for gene transfer [21]. This finding further corroborates the observation that the genetic manipulation of an unexplored cyanobacterium can sometimes be an insurmountable task and that a genetic system efficient for one species may indeed be ineffective in a closely related one.

In the investigated *Chroococcidiopsis* strains gene transfer via conjugation was more efficient than electroporation, supporting the observation that the extracellular nucleases produced by cyanobacteria reduce transformation efficiencies. Indeed, in the investigated *Chroococcidiopsis* strains the presence of extracellular nucleases was revealed by clearing zones in agarized growth medium containing DNA-methyl green [21].

To assess the suitability of desert strains of *Chroococcidiopsis* for gene transfer, different plasmids known to replicate in cyanobacteria were used. Plasmids derived from the IncQ group failed to replicate in any of the investigated

strains. By contrast, pDU1-based plasmids were shown to be autonomously maintained in three strains of *Chroococcidiopsis*. Thus these shuttle plasmids offered the possibility of using standard recombinant DNA techniques easily performed in *Escherichia coli* and the achievement of gene transfer into *Chroococcidiopsis*.

The observation that the conjugative transfer of pDU1-based plasmids into *Chroococcidiopsis* sp. strains is unaffected by the absence of helper plasmids suggested that in the conditions used, their restriction modification system does not prevent their genetic manipulation [21]. To date, among cyanobacteria displaying a remarkable tolerance to desiccation, such as *Nostoc commune*, desert strains of *Chroococcidiopsis* are the only ones suitable for genetic manipulation [22].

3.2. Reporter genes in *Chroococcidiopsis*

To achieve highly sensitive measurements of the transcription level of a given gene the expression of the reporter gene *luxAB*, encoding a bacterial luciferase, under the constitutive promoter P*psbA,* derived from the chloroplast of *Amaranthus hybridus* was attempted in desert strains of *Chroococcidiopsis* [21]. The P*psb*A-*luxAB* expression was visualized *in vivo* by recording for a few minutes the bioluminescence of transformed colonies after the addition of the luciferine needed for the oxidate reaction catalyzed by the luciferase (Fig. 1a). The bioluminescent colonies of transconjugants of *Chroococcidiopsis* shown in Fig. 1b, were obtained according to the triparental mating procedure, performed by spotting on filters on top of cyanobacterial agarized growth medium, an aliquot of *E. coli* mobilizer and donor strains along with the cyanobacterium. The possibility of using luciferase-based approaches in *Chroococcidiopsis* will allow the monitoring of its responses to environmental changes, as previously reported for other cyanobacteria [23].

More recently, a genetic system was developed in order to use the autofluorescent green fluorescent protein (GFP) as a reporter system in *Chroococcidiopsis*. This protein has an advantage over the luciferase in being easily detected in epifluorescence microscopy and in not requiring a substrate [24]. Therefore he *luxAB* gene carried in a previously employed pDU1-based plasmid was replaced with a *gfp* gene (Fig. 1c). The availability of such a genetic system for in vivo imaging in *Chroococcidiopsis* has a potential application in monitoring gene expression as well as visualization of GFP-tagged proteins. In addition GFP-based shuttle plasmids provide an easy and sensitive way to demonstrate gene transfer into a given cyanobacterium.

4. Means of mutagenesis

Mutations are central to genetics, either to identify unknown genes involved in a particular process, or to elucidate the function of known genes. Mutations can be generated physically (e.g. with UV light), chemically (e.g. with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine), or biologically (e.g. with transposons or insertional mutagenesis). Among the latter random transposon mutagenesis has often facilitated the search for mutants since cyanobacteria have multiple copies per cell of their genome [13]. Transposons have the advantage of tagging the genomic site of the mutation, so facilitating recovery of the wild-type form of the mutated locus; it is also preferable to chemical and UV mutagenesis because it yields an antibiotic-resistant marked mutant population [12, 13].

A given gene can be mutagenized by homologous recombination. Single-crossover homologous recombination inactivates the gene of interest only if the recombined fragment lacks the start and end of the transcriptional unit [12]. To perform mutagenesis *via* double recombination there is the necessity for a whole gene interrupted by an antibiotic resistance and also for a lethal gene, such as the *sacB*; this is to avoid merodiploids which carry a mutate and a wild-type gene copies, both of which in turn originate from single-crossover recombination [25]. In addition, due to the presence of multiple copies of the genome, the introduced mutation will be heterozygous, until replication and segregation of the genomes produce cells that are homozygous for the mutation [13].

4.1. Gene inactivation in *Chroococcidiopsis*

The tractability in desert strains of *Chroococcidiopsis* of transposon mutagenesis was investigated by evaluating the capability of Tn*5* to transpose into this cyanobacterium. Cyanobacterial cells were mated with an *E. coli* strain harbouring a mobilizable plasmid with a Tn5 system and a promoterless *luxAB*. Colonies of transconjugant were obtained according to the plating mating procedure in which a mixture of *E. coli* donor strain and the recipient cyanobacterium are spread on filters on top of cyanobacterial agarized growth medium. As shown in Fig. 1d, among the transconjugants colonies some show different bioluminescent signals due to the various transcriptional fusions to *Chroococcidiopsis* genome realized upon the transposition. This provides evidence that transposon Tn5 can be used to generate transcriptional fusions of promoterless *luxAB* into *Chroococcidiopsis* genome, thus making it possible to use this tool to identify genes responsive to environmental shifts by monitoring the increase or decrease of the luminescence of transposon-generated colonies.

The inactivation of a given gene was first attempted in *Chroococcidiopsis* by using a both-ends truncated fragment of the *ftsZ* gene cloned into a mobilizable plasmid unable to replicate inside this cyanobacterium [26]. The integration by

single crossover of such a plasmid into the host genome produced two mutated copies of *ftsZ*, with the whole plasmid in-between. While it was not possible to select cells which segregated the mutation, since *ftsZ* is an essential gene, heteroplasmic *ftsZ* mutants showed an aberrant phenotype ascribable to the effect of the partial inactivation of the investigated gene [26].

5. Genome sequencing

The future availability of the whole genomic sequence of at least one desert strain of *Chroococcidiopsis* (Steve Pointing, personal communication) will provide the opportunity to monitor global changes at a transcriptional and translational level, thus greatly contributing to unravelling the molecular bases underlying the extreme resistance of *Chroococcidiopsis* to desiccation and radiation [27]. In addition the availability of gene sequences will support *in vivo* imaging of selected proteins fused to proper reporter genes. Finally, the currently developed genetic tools along with the genome data will facilitate the biotechnological exploitation of this extreme-tolerant cyanobacterium, for example in the field d of air-drying and storage of biological materials.

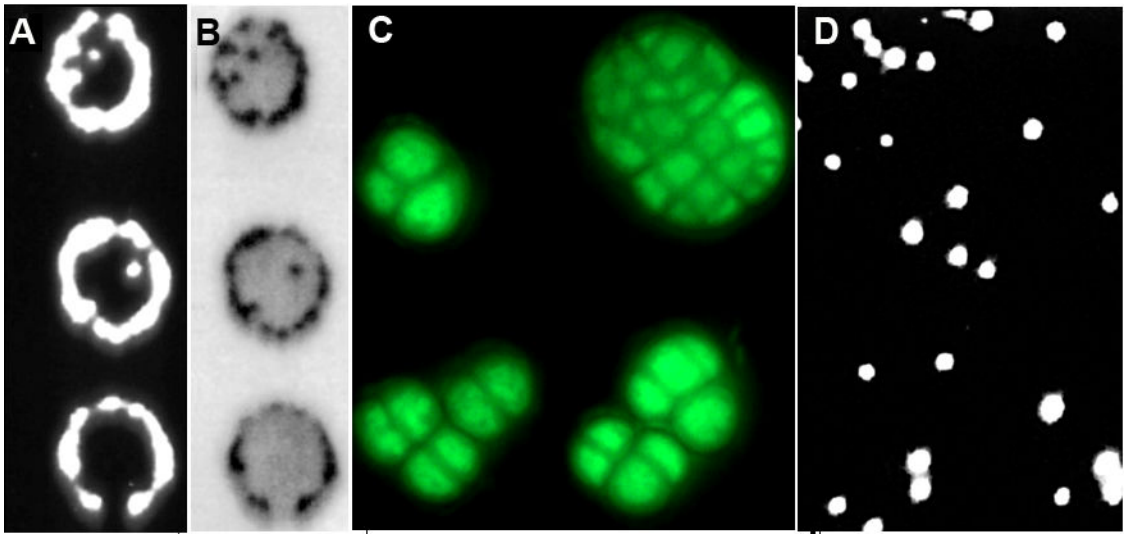

Fig. 1. A-B: Biolumescence of P*psb*A-*luxAB* in *Chroococcidiopsis* colonies. C: Fluorescence of *Chroococcidiopsis* cells expressing a *gfp* gene. D. *Chroococcidiopsis* colonies showing transcriptional fusions of the promoterless *luxAB* into its genome.

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