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An expression system for regulated protein production in *Synechocystis* sp. PCC 6803 and its application for construction of a conditional knockout of the ferrochelatase enzyme

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ABSTRACT

Synechocystis sp. PCC 6803 is a model organism for the study of photosynthetic processes. Methods to genetically manipulate this bacterium are essential to investigate these processes and to evaluate potential biotechnological applications. We developed a vector for controllable expression of proteins using a platform for stable integration of the expression cassette into the genome. The respective gene is translationally fused to the promoter of the *petJ* gene encoding cytochrome c_{553} that is repressed by copper. Maximal expression from this promoter is achieved under copper depletion, whereas normal copper concentrations in standard medium lead to low expression rates. We show here the application of this system for construction of a conditional knockout mutant for the ferrochelatase, which is an essential enzyme in heme biosynthesis. Using different amounts of copper in the medium we were able to control the amount of ferrochelatase in the cell resulting in a varying expression of the phenotype.

1. Introduction

Synechocystis sp. PCC 6803 (hereafter *Synechocystis* 6803) is a fast growing unicellular cyanobacterium performing oxygenic photosynthesis. The complete sequence of its genome has been published in 1996 (Kaneko et al., 1996). This was one of the main reasons for the development of this cyanobacterium into a model organism for the study of photosynthesis and gene regulation. *Synechocystis* 6803 was shown to be naturally transformable (Grigorieva and Shestakov, 1982). The uptake of foreign DNA using type IV pili (Yoshihara et al., 2001) is followed by its incorporation into the *Synechocystis* 6803 genome by homologous recombination, almost always as double recombination (Williams, 1988). Effective methods for recombinant expression of foreign genes as well as overexpression of its own genes together with the availability of complete genome sequence make this cyanobacterium an attractive model organism for biotechnical applications and fundamental research. There are several applications, where regulable expression of genes in the cyanobacterial host is useful (reviewed in Wilde and Dienst, 2011). In order to exclude that a specific deletion of a gene has polar effects on the expression of up- or downstream genes, complementation studies have to be performed. Other implementations include studies on phenotypical effects of overexpressed genes and production of proteins in *Synechocystis* 6803 cells. Overproduced proteins can be isolated using tags previously added to the protein of interest. However, the potential of expressing genes in this system can be extended to more applied goals. Several properties of cyanobacteria make them attractive candidates for biotechnological applications. They are easily cultivable in large quantities in media consisting solely of water (also sea water), some minerals, carbon dioxide, and sunlight. Therefore, cyanobacteria could provide an attractive alternative for production of high-value products, but also fuels, thus preventing disadvantages associated with the conventional conversion of plant biomass into biofuels (Ducat et al., 2011; Angermayr et al., 2009). Applications include the introduction of new biochemical pathways from other organisms into *Synechocystis* 6803 or to alter metabolic flow through existing biochemical pathways (e.g. Lindberg et al., 2010).

Overexpression of genes can be achieved by using a self-replicating plasmid or suicide vectors that contain an integration platform allowing the insertion of the foreign gene equipped with a suitable promoter into a neutral site of the chromosome. Neutral sites are thought to be sequences where DNA fragments can be inserted without generating an altered phenotype. Such a sequence can be a redundant or a silent gene, for example the widely used

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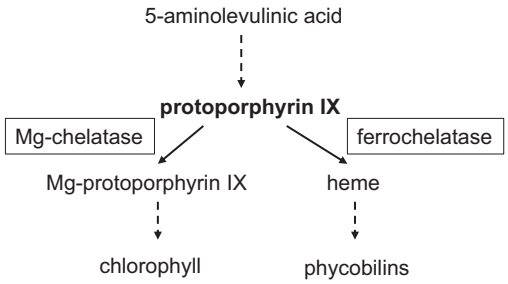

Fig. 1. Scheme of tetrapyrrol biosynthesis. Cyanobacteria, algae and plants synthesize tetrapyrroles via a common branched pathway. The precursor 5-aminolevulinic acid is subsequently converted in several steps to protoporphyrin IX, the last common precursor for both chlorophyll and heme biosynthesis. Insertion of Fe^{2+} into this porphyrin by ferrochelatase leads to heme and by further processing to bilins, whereas insertion of Mg^{2+} by magnesium chelatase leads to Mg-protoporphyrin IX, the first intermediate of chlorophyll synthesis.

psbA2 gene (Lagarde et al., 2000). Another approach is the use of intergenic regions between protein coding genes as integration platforms. Since there is increasing evidence for the presence of regulatory RNA genes these intergenic regions should not be considered a priori as neutral (Mitschke et al., 2011). In addition, insertion of foreign DNA into untranslated 5′ or 3′ regions can lead to transcript destabilization or alterations in promoter structures.

We describe here the construction of a suicide vector for the stable integration of foreign and own genes into the *Synechocystis* 6803 chromosome and its expression using a tightly regulated promoter. Inducible expression of the ferrochelatase (FeCH) gene *hemH* is shown as an example for the use of this expression vector to study the function of an essential gene in knockout mutants. A similar approach has been shown for the gene encoding the RNA subunit of ribonuclease P, which is essentially involved in tRNA processing (Tous et al., 2001).

The FeCH is an essential enzyme that catalyses the insertion of Fe^{2+} into the protoporphyrin IX macrocycle (reviewed in Tanaka and Tanaka, 2007). This step marks the start of the heme biosynthetic pathway (Fig. 1). Heme itself is cleaved by heme oxygenase producing biliverdin, the precursor for the synthesis of phycobilins. In cyanobacteria this step is very important, as phycobilisome containing organisms use phycobilins as the major chromophore for light harvesting in addition to chlorophyll. Because heme is also an essential cofactor for cytochromes, representing electron carriers that are crucial for the electron transfer chain, inactivation of the *hemH* gene should not be possible in cyanobacteria. However, *Synechocystis* 6803 contains several chromosomes per cell (Labarre et al., 1989; Griese et al., 2011). Thus, it is possible to obtain not completely segregated mutant cells, where only a part of chromosomal copies harbor a mutated version of an essential gene. In order to generate a complete knockout of the ferrochelatase gene, *hemH* was first ectopically expressed under control of a regulated promoter followed by segregation of mutated version of the entire *hemH* locus under inducible conditions.

2. Materials and methods

2.1. Strains and growth conditions

The *Synechocystis* 6803 (GT) strain was a gift of M. Tichy (Institute of Microbiology, Academy of Sciences, Třeboň, Czech Republic). This sub-strain, originally decribed by Williams (Williams, 1988), was routinely grown in liquid BG-11 medium (Rippka et al., 1979) supplemented with 10 mM TES at 30 °C and at 50 μmol photons $m^{-2}\,s^{-1}$ white light (Philips TLD Super 80/840). Cells were grown on plates with 0.75% agar (Difco). Antibiotics were added

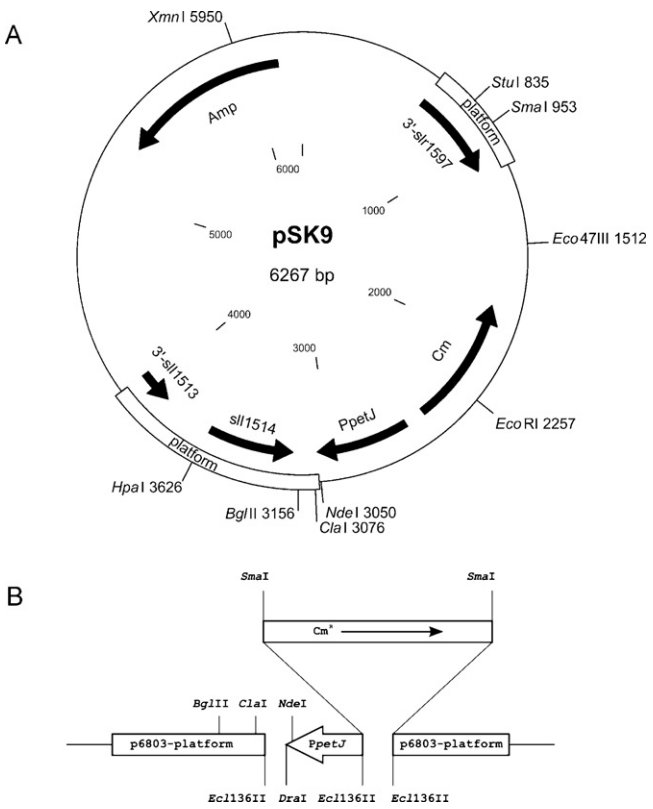

Fig. 2. Construction of the expression vector pSK9. (A) Map of the expression vector. The introduced NdeI site contains the start codon for the gene product of interest. Respective genes will be integrated into the genome of *Synechocystis* 6803 under control of the copper controlled promoter of the *petJ* gene using an integrative platform between *sll1514* and *slr1597* (B).

at concentrations of 40 μg mL^{-1} for kanamycin and 7 μg mL^{-1} for chloramphenicol. *Synechocystis* 6803 cells were grown in BG11 medium with the copper concentrations indicated. For growth in copper-free medium $CuSO_4$ was omitted from the trace metal mix and cells were washed in copper-free medium for two times.

2.2. Construction of psk9 vector

A 490-bp DNA fragment carrying a promoter region of the *petJ* gene was amplified by PCR from genomic DNA of *Synechocystis* 6803 using primers P1 (5′-AATTTAAACATATGTTCTCCTTTCAAGG-3′) and P2 (5′-TTGAGCTCATCGGGGGCTGTGTTGG-3′). The primers were designed with addition of restriction sites to their 5′-ends (DraI and NdeI for P1 and Ecl136II for P2). The introduced NdeI site contains the start codon for the gene product of interest. The PCR product was cleaved by DraI and Ecl136II and cloned into the Ecl136II site of the integrative platform (Sergeyenko and Los, 2003). The pUCCm (Department of Genetics, MSU collection) was used as a source of chloramphenicol resistance cassette. This plasmid is a derivative of the pUCGm (Schweizer, 1993) in which Ecl136II-fragment that confers gentamycin resistance was replaced by BsaAI-fragment containing *cat* gene from pACYC184 (Rose, 1988). The chloramphenicol resistance cassette from pUCCm was digested with SmaI and inserted into the Ecl136II site that remained on the border of the *petJ* promoter region and the integrative platform. The *cat* gene in the resultant plasmid is transcribed in opposite direction to the *petJ* promoter (Fig. 2A and B). The vector was named psk9 and the sequence was submitted to GenBank with Database ID: JQ322997 (pVZExCu).