Production of green fluorescent protein by the methylotrophic bacterium Methylobacterium extorquens

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Abstract

The production of green fluorescent protein (GFP) in Methylobacterium extorquens was studied by creating four different constructs using pJB3KmD, pRK310 and pVK101 vectors, as well as pLac and soluble methane monoxygenase (sMMO) promoters. Plasmids were introduced into the cells by electroporation. Expression of GFP by selected clones was evaluated by growing cells in complex or defined media. The use of pRK310 as an expression vector containing the lacZ promoter resulted in a 100-fold increase of GFP production when compared to cells containing the pLac-GFP-pJB3KmD construct. Higher production of GFP was observed also in cells containing pLac-GFP-pRK310 and pmmoX-GFP-pVK101 constructs. While the transcriptional regulation of the smmo gene in Methylosinus trichosporium OB3b is known to be copper-dependent, expression of GFP by M. extorquens clones harboring pmmoX-promoters was not strongly controlled by the presence of copper in the medium. The production of GFP was generally constant throughout the growth of M. extorquens carrying the pLac-GFP-pRK310 construct. GFP yields varied between 850 and 1000 W g of GFP g biomass \(^{-1}\). However, the yield of GFP in cells carrying pmmoX-GFP-pVK101 was somewhat reduced after the mid-exponential phase of growth. © 2000 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

Methylotrophic bacteria are a group of microorganisms that can utilize one-carbon (C\(_1\)) compounds more reduced than carbon dioxide as a source of carbon and energy. Formaldehyde, an intermediate in the oxidation of reduced C\(_1\) compounds is incorporated into cell carbon via the serine pathway or can be further oxidized in a series of reactions to \(\text{CO}_2\) generating energy in the form of reducing equivalents.

*Methylobacterium extorquens* ATCC 55366 is a pink-pigmented facultative methylotroph isolated from a hydrocarbon-contaminated sandy soil [1]. The growth of this bacterium in a fed-batch fermentation system developed by Bourque et al. [2] resulted in the cultivation of very high cell densities using a relatively cheap substrate, methanol, for the production of poly-\(\beta\)-hydroxybutyrate.

The ability to produce high biomass densities in fermentors, combined with the newly acquired genetic information obtained from the genome sequencing of *M. extorquens* [3], renders this microorganism extremely interesting as a potential expression system for recombinant proteins and for the production of industrially important bulk chemicals. In order to achieve these objectives, it is essential to identify efficient cloning vectors and promoters for introducing new genes into *M. extorquens*. In this study we have constructed and compared different expression systems in *M. extorquens*. The reporter *gfp* (green fluorescent protein) gene was cloned into several expression vectors. GFP from the jellyfish *Aequorea victoria* has been shown to be a powerful reporter for the analysis of gene
expression, as well as protein localization in a wide variety of experimental systems [4].

2. Materials and methods

2.1. Bacterial strains, plasmids and growth conditions

M. extorquens ATCC 55366 [1] was grown as described previously [2]. Electro-competent cells of M. extorquens were prepared by the method of Toyama et al. [5] with slight modifications. Cells were grown in CHOI medium (containing 1% v/v methanol) until the culture reached an OD_{600} \approx 0.6–0.8. Cells were harvested by centrifugation (1800 \times g, 10 min, 4°C) and washed twice with ice-cold sterile 10% (v/v) glycerol solution. The cell suspension was concentrated 10-fold in 10% glycerol, dispersed in 400-μl aliquots and kept at −80°C. Electro-competent cells (100 μl) were mixed with DNA solution (500 ng) in a 0.2-cm gap cuvette chilled on ice. Electroporation was carried out using a Gene Pulser (Bio-Rad) with the following parameters: 2.5 kV, 400 μF, to a final field strength of 12.5 kV cm⁻¹. After cells had been pulsed, 1 ml of ice-cold sterile Luria–Bertani low salts (LBLS) medium was added to the cuvette immediately, and the cell suspension transferred into a test tube, and then incubated at 30°C for 24 h. Transformed clones were selected in LBnS (Luria–Bertani without NaCl) agar medium with appropriate antibiotics (kanamycin, 20 μg ml⁻¹; tetracycline, 20 μg ml⁻¹). Escherichia coli DH5α (Life Technologies Gibco BRL) was cultivated at 37°C in LBLS broth or agar plates. Plasmids in E. coli were selected with ampicillin (100 μg ml⁻¹), kanamycin (50 μg ml⁻¹) or tetracycline (20 μg ml⁻¹). Information on the plasmids used in this study is given in Table 1.

2.2. Construction of plasmids

In vitro DNA manipulation for cloning in E. coli was performed as described by Sambrook et al. [6]. The strategy used to create different GFP-carrying plasmids is represented in Fig. 1. The set of primers used were: (a) GFP/BamH1.2 (5'-GAA TCG GGA TTC GTA CAG TTC ATC CAT GC-3'; BamHI restriction site underlined) and RBS/PstI.2 (5'-AAC AAA CTG CAG AAA ATG TTG GTA CAG TTG TAA CTT TAA GAA GAG-3'; PstI restriction site underlined); and (b) RBS/MluI (5'-CAC GAC CCG TTG AAA TAA TTT TGT TTA ACT TTA AGA AGG-3'; MluI restriction site underlined) and GFP/XbaI (5'-TGC TCT AGA TCA GTT GTA CAG TTC ATC CAT GC-3'; XbaI restriction site underlined). The polymerase chain reaction conditions in both cases were: hot start at 94°C for 2 min and then 30 cycles of amplification (94°C, 30 s; 55°C, 30 s; 72°C, 30 s) followed by a final extension at 72°C for 10 min.

2.3. Detection of GFP expression in M. extorquens

Selected clones of M. extorquens carrying GFP constructs were grown in LBnS or CHOI medium containing 1% methanol and the appropriate antibiotic (kanamycin, 20 μg ml⁻¹; tetracycline, 20 μg ml⁻¹) at 30°C, 250 rpm. After 72 h of incubation, cells were harvested by centrifugation and washed twice with sterile, deionized water. Cells were resuspended in 700 μl of water and two 100-μl aliquots were dispensed into 96-well plates. The remaining 500 μl was used to determine cell dry weight. Cells harboring pJB3KmD, pRK310 or pVK101 were used as control, and their fluorescence was subtracted from values obtained with cells harboring plasmids containing the gfp gene.

GFP production was determined in M. extorquens cells growing in CHOI medium. Cells were initially grown on 50 ml CHOI medium until the end of exponential phase (OD_{600} \approx 0.8). A 2% inoculum was then used to start the growth curve on 200 ml CHOI medium during which samples were taken for measurement of OD_{600}, fluorescence and dry weight.

Fluorescence of cell suspensions was determined in a Cytofluor 2300 System (Millipore) under excitation and emission wavelengths of 485 and 530 nm, respectively. The concentration of GFP was calculated based on a lin-

Table 1

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Source</th>
</tr>
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<tbody>
<tr>
<td>pRK310</td>
<td>cloning vector, lacZ', oriV, oriT, Te', 19 kb</td>
<td>[5]</td>
</tr>
<tr>
<td>pLac-GFP-pJB3KmD</td>
<td>pMTL1000 with 0.78-kb insert containing GFP coding sequence from pQBI63</td>
<td>this study</td>
</tr>
<tr>
<td>pLac-GFP-pRK310</td>
<td>pRK310 with ( \sim ) 0.78-kb insert containing GFP coding sequence from pQBI63</td>
<td>this study</td>
</tr>
<tr>
<td>pmmx-X-GFP-pRK310</td>
<td>pRK310 with ( \sim ) 9.5-kb insert containing sMMO and GFP coding sequence from pMTL1000</td>
<td>this study</td>
</tr>
<tr>
<td>pmmx-X-GFP-pVK101</td>
<td>pVK101 with ( \sim ) 9.5-kb insert containing sMMO and GFP coding sequence from pMTL1000</td>
<td>this study</td>
</tr>
</tbody>
</table>

Ap', Km', Te' denote resistance to ampicillin, kanamycin and tetracyclin, respectively.

*Accession Databank No. U75323.
ear relationship between concentration and fluorescence determined for solutions of purified of GFP (Quantum Biotechnologies). GFP yield is reported as GFP concentration (µg) per unit of dry weight (g).

2.4. Determination of Cu concentration in solution

Cells grown in the presence of Cu were harvested by centrifugation and the supernatant was collected for Cu analysis. 0.5 ml of concentrated H$_2$SO$_4$ (93%) was added to 9.5 ml of supernatant in order to maintain the pH lower than 2.0 (thus preventing Cu precipitation) for Cu ions analysis using inductively coupled plasma-atomic spectrometer (ICP-AS; Thermo Jarell Ash, Trace Scan). The result was corrected with the appropriate dilution factor and referred to as the final soluble Cu concentration. The total Cu concentration added to the medium at preparation was determined likewise by adding 5% (v/v) concentrated H$_2$SO$_4$ to the medium and then analyzing using ICP-AS.

3. Results and discussion

3.1. Efficiency of transformation of M. extorquens by electroporation

An essential step in achieving the expression and stability of heterologous genes in host methylotrophs is through the use of suitable broad-host-range vectors. The plasmids used here, pJB3KmD, pRK310 and pVK101, are derived from broad-host-range vectors which were developed for Gram-negative bacteria [7–9]. Table 2 shows the time constant produced, as well as the efficiency of transformation for the different constructs used to transform M. extorquens. An overall improvement was observed on the efficiency of transformation of each plasmid in the following order: pJB3KmD < pRK310 < pVK101. While the time constant values were within the range obtained by Toyama et al. [5] when applying similar electroporation conditions, the efficiency of transformation observed in this study for pRK310 (∼10$^3$ cells µg$^{-1}$ DNA) was at least
two orders of magnitude lower than that obtained by them. This value was, however, close to that obtained by Ueda et al. [10] when they electroporated \textit{M. extorquens} with pLA2917. Such differences might be due to the strain differences or diverse conditions used for preparing electropotent cells, as well as due to specific electroporation conditions such as the time constant produced after each energy discharge. Nonetheless, the transformation efficiencies obtained in this study are high enough for practical use in genetic manipulation. The cosmid pVK100 (which resembles pVK101 except for the presence of the \textit{cos} factor in pVK100) was shown to be mobilized from \textit{E. coli} strains into \textit{M. extorquens} AM1 (previously known as \textit{Pseudomonas} sp. AM1) by conjugation at frequencies of $10^{-1}$ to $10^{-2}$ [11], lower than the ones observed in the present study. There are very few reports in the literature on the use of electroporation as a means of introducing DNA into \textit{M. extorquens}. Although conjugation has been the preferred technique for transforming methylotrophic bacteria, electroporation was proven here to be a faster and less laborious technique.

### 3.2. GFP production under the control of the \textit{lacZ} promoter

The \textit{gfp} gene used in this study originated from a modified construct of the wild-type GFP [12]. Its transcription is under the regulation of the \textit{lacZ} promoter or the soluble monooxygenase gene cluster promoter \textit{mmoX} [13]. The \textit{lacZ} promoter has been successfully used for the expression in GFP by several bacteria [14]. The fusion of \textit{lacZ} regulative elements in constructs containing mosquitocidal endotoxins gene (\textit{cryIVB}) from \textit{Bacillus thuringiensis} led to a significant increase of the \textit{cryIVB} gene expression in the obligate methylotroph \textit{Methyllobacillus flagellatum} [15]. However, the absence of the \textit{lacI} gene gives rise to a constitutive phenotype and thus the \textit{lac} promoter is induced constitutively even without inducers [16].

The \textit{lac} promoter was recognized by \textit{M. extorquens} in the present study, in accordance with previous findings [5]. It was also found to be constitutively induced (data not shown). An interesting observation arises from the comparison between the fluorescence produced by clones of \textit{M. extorquens} carrying the GFP gene in either pJB3KmD or pRK310 (Fig. 2). The use of the latter led to at least a 100-fold improvement in the amount of GFP produced by each clone.

An important difference was also observed in the amount of GFP produced by clones growing in either LB or CHOI medium (Fig. 2). Independently of the construct used, an improvement of at least 30\% in the yield of GFP was obtained when cells grew in CHOI medium. The CHOI medium was described as the ideal medium to produce high biomass yields of \textit{M. extorquens} [2]. The hypothesis that nutrients lacking in the culture medium may interfere in the production of GFP or in its chromophore activity [17] should be further investigated in order to explain the significant difference in yields of GFP depending on the medium used.

### 3.3. GFP production under the control of \textit{mmoX} promoter

In this study, we transformed \textit{M. extorquens} with constructs containing the \textit{gfp} gene under the control of \textit{mmoX} promoter present in the soluble methane monooxygenase (sMMO) operon of another methylotrophic bacterium, \textit{Methylosinus trichosporium} OB3b. In this microorganism, sMMO catalyzes the oxidation of methane to methanol. The transcriptional regulation of the \textit{smmo} gene is known to be copper-dependent, sMMO is expressed only under conditions in which the copper-to-biomass ratio is low. This allows a strict control of the expression of the gene under its promoter by controlling the concentration of Cu in the medium [13].

Transformed \textit{M. extorquens} cells were grown in defined medium (CHOI) so that the interference of varying con-

![Fig. 2. GFP production by \textit{M. extorquens} in LB or CHOI media. Bars represent the error deviation within two independent experiments.](image-url)
centrations of Cu$^{2+}$ in the production of GFP could be determined (Fig. 3). It was found that the promoter is recognized by *M. extorquens* therefore allowing the expression of GFP. The increase on the initial Cu$^{2+}$ concentration (from 0 to 57 $\mu$M) did not interfere in the cell growth (as observed by the final dry weight). However, expression of GFP by clones carrying both *pmnoX*-GFP-pRK310 and *pmnoX*-GFP-pVK101 was not strongly controlled by the presence of Cu in the medium and repression was observed to some extent when the initial concentration of Cu in the medium was increased up to 57 $\mu$M.

Copper speciation in the medium and its effect on the activity of sMMO is also relevant to understanding the *smmo* regulation. Morton et al. [18] found no detectable sMMO activity when $>2.63$ $\mu$M Cu g protein$^{-1}$ was present. Moreover, different sMMO activities were observed, depending on the type of Cu complex present, which could be explained by the inability of cells to actively transport Cu complexes into the cells, thus reducing Cu bioavailability. In the present study, the analysis of soluble Cu present in the medium at the end of cell growth experiments revealed that between 11 and 21% of the Cu added to medium was present in its free form (Fig. 3). The remainder Cu was probably precipitated as oxides, hydroxides or ligand complexes, or accumulated by the cells. While the effect of different species of Cu could affect the regulation of *pmnoX*, the final concentrations of free Cu in the medium should be high enough to totally inhibit the *pmnoX* expression. However, a reduction of 41 and 33% of GFP production was observed when the final Cu concentration was 2.4 and 3.9 $\mu$M Cu mg biomass$^{-1}$ (for the clones 1–3 and 3–63, respectively).

The growth of *M. extorquens* carrying the *pmnoX*-GFP-pVK101 construct (in the absence of Cu) (Fig. 4) showed that the yield of GFP during growth reached its maximum at mid-exponential phase (about 700 $\mu$g of GFP g biomass$^{-1}$) and decreased as the culture reached the early stationary phase of growth (350 $\mu$g of GFP g biomass$^{-1}$ at stationary phase). Since the fluorescence of cells remained constant in the stationary phase, this suggests that the reduced GFP yield observed may be due to the cessation of GFP production during this phase. There could be several factors related to the growth conditions of *M. extorquens* carrying *pmnoX*-GFP-pVK101 (including the O$_2$ or redox potential limitations which are known to dramatically affect the maturation of GFP [17]) that could explain the end of GFP production at the stationary phase.

GFP has now been used as a model heterologous protein in order to identify suitable vectors as well as efficient promoters for *M. extorquens*. pRK310 and pVK101 constructs containing Lac and *mmoX* promoters are valuable.
expression systems for GFP and the expression of other industrially more important genes in this bacterium should now be more easily accomplished. Also, since these vectors are known to be maintained in methanotrophs [19], the expression of GFP, vis-à-vis its applications in gene regulation studies in these microorganisms, is currently being carried out in our laboratory using the constructs described here.

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References


