Efficient Expression of a Carbon Starvation Promoter Activity Under Nutrient-Limited Chemostat Culture

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Abstract The promoter region of a carbon starvation gene isolated from *Pseudomonas putida* was cloned and analyzed for its potential use for *in situ* bioremediation and bioprocessing. We constructed a recombinant plasmid pMKD101 by cloning the 0.65 kb promoter region of the gene into the promoter proving vector, pMK301, which contains the *lacZ* for β-galactosidase activity as a reporter gene. pMKD101 was transformed into the wild-type *P. putida* MK1, resulting in *P. putida* RPD101, and analyzed for β-galactosidase activity under different culture conditions. When RPD101 was grown on the minimal medium plus 0.1% glucose as a sole carbon source in batch cultures, β-galactosidase activity was found to be 3.2-fold higher during the stationary phase than during the exponential phase. In chemostat cultures, β-galactosidase activity was found to be 3.1-fold higher at the minimal growth rate (dilution rate=0.05 h^{-1}) than at the maximal growth rate (dilution rate=0.173 h^{-1}). The results suggest that a carbon starvation promoter can be utilized to maximize the expression of a desired gene under nutrient limitation.

Key words: Starvation promoter, bioremediation, bioprocessing, *Pseudomonas putida*

In the industrial fermentation process, useful bio-product formation usually relies on the rapid growth of bacteria with rich nutrients. This process, however, hampers the quality of bioreactor, where bacteria are immobilized due to a large biomass increase [6]. A promising technique to circumvent this situation would be to minimize the bacterial growth, while maximizing a desired biochemical activity. The technology to utilize starvation promoters would be a suitable candidate for this purpose. Starvation promoters drive the transcriptional expression of starvation genes, which are induced at the onset of nutrient starvation [10]. Therefore, by placing a desired gene under the control of a starvation promoter through the recombinant DNA technology, a bacterial system that restricts both the amounts of nutrients and biomass while constantly expressing a high level of enzyme activity during restricted growth can be achieved.

Such technology is also desirable in *in situ* bioremediation. Biostimulation is one of the currently used approaches for bioremediation [4, 15], by feeding nutrients to the contaminated sites to stimulate the growth of indigenous bacteria. However, this technology has various problems. For example, in addition to cost and time, there are technical hindrances to introduce a large quantity of nutrients. The process can also result in plugging of the pore for nutrient passage, due to large biomass increase, hampering further progress [10].

Matin and co-workers [11] have pioneered the study of this endeavour. They have designed a bacterial system for the degradation of trichloroethylene, using one of the *E. coli* starvation promoters [11]. The designed *E. coli* strain expressed a starvation promoter-driven gene under both a glucose-starved batch culture and a glucose-limited chemostat culture. However, *E. coli* is not suitable for *in situ* application, since it is not indigenous in nature. *Pseudomonas* species are ubiquitous in our environment, in which they frequently encounter nutrient-limited conditions and are well known for their ability to degrade a variety of aromatics [14, 16]. Previously, Kim *et al.* [8] have cloned and characterized several starvation promoters from *Pseudomonas putida*, which is an environmentally relevant bacteria, hence suitable for the test for *in situ* efficacy. One of these promoters, which drives the gene *flhF* involved in flagellar biogenesis, was used to construct the starvation promoter-driven vector in this study. The resultant vector was reintroduced into *P. putida*, and the promoter activity was analyzed in a glucose-limited chemostat culture.

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Table 1. Strains and plasmids used in this study.

| Bacterial strains or plasmids	Relevant characteristics	References or sources
Strains		
E. coli		
MC1061	*hsdR2 hsdM⁻ hsdS⁻ araD139 Δara-leu) Δlac galE15 galK16 rpsL mcrA*	[8]
CC118λpir	*Δ(ara-leu) araD ΔlacX74 galE galK phoA20 thi-1 rpsE rpoB argE(Am) recA1 λpir* phage lysogen; Rif⁻	[3]
DH5α	*F'/endA1 hsdR17(rk⁻mk⁺)supE44 thi-1 recA1 gyrA* (Nal⁻) *relA1 Δ(lacZYA-argF)*$_{U169}$*(m80lacZΔM15)*	This laboratory
Pseudomonas putida		
MK1	Derivative of ATCC 12633; Rif⁻	[8]
RPT101	MK1 harboring pMKT101	This study
RPD101	MK1 harboring pMKD101	This study
RPT301	MK1 harboring pMKT301	This study
Plasmids		
pUT mini-Tn5::Tc	Apʳ Kmʳ; delivery plasmid for mini-Tn5::Tc	This laboratory
pMKU101	pUC19 with 0.65-kb *EcoRI-PstI* inserted; Apʳ	[8]
pMKT101	pMKU101 with 2.1-kb Tc cassette inserted at *Hind*III site; Apʳ, Tcʳ	This study
pMKD101	pMK301 with *EcoRI-Hind*III fragment from pMKT101, which contains deleted promoter region and 2.1-kb Tc cassette; Apʳ, Tcʳ	This study
pMK301	*lacZ1* promoter probe vector; Apʳ	[8]
pMKT301	pMK301 with 2.1-kb Tc cassette inserted at *Hind*III	This study

All the strains and plasmids used in this study are listed in Table 1. Luria-Bertani (LB) and M9 minimal media were prepared as described previously [8]. Fifty and 25 μg of tetracycline per ml were used for *P. putida* and *E. coli*, respectively. 5-Bromo-4-chloro-3-indolyl-β-D-galacto pyranoside (X-Gal, 50 μg/ml) was used to detect β-galactosidase production on plates.

The parental plasmids, including pMKU101, pUT-mini Tn5::Tc, pMKT101, and pMK301, were used to construct the plasmid pMKD101. The pMKU101 containing the 0.65 kb starvation promoter region was digested with *Hind*III, and then a 2.1 kb *Hind*III fragment containing the tetracycline-resistant gene from the pUT-mini *Tn5*::Tc was cloned into pMKD101 to construct pMKT101. The pMKT101 was first cut with *EcoRI* and then partially digested with *Hind*III. The 2.75 kb DNA fragment containing a promoter region and Tc-resistant gene was isolated and cloned into the promoter probing vector pMK301, which was cut with the same enzymes. All genetic techniques such as plasmid DNA isolation, agarose gel electrophoresis, isolation and purification of DNA fragment from agarose gels, preparation of competent cells, restriction endonucleases digestion, ligation of DNA with T4 DNA ligase, calf intestinal alkaline phosphatase treatment, and transformation were performed by standard procedures [9].

P. putida RPD101 were grown at 37°C in a 3-l bioreactor (BIO-TRON; BioG-M) with a working volume of 1 l with M9 medium plus 0.1% glucose. The aeration rate was 0.1 l/min, and agitation was maintained at 500 rpm. The pH remained at 7.0 throughout the cultivation. Plasmid

stability studies were carried out by a replica plating technique on tetracycline selection plates. In batch cultures, 1 ml of each sample was taken at every one hour interval, and its optical density at 660 nm and β-galactosidase activity were analyzed. In continuous cultures, samples were withdrawn from the culture at different dilution rates and analyzed for β-galactosidase activity. The assay for β-galactosidase activity was performed as described by Miller [12].

The plasmid pMKD101 was constructed by cloning the 0.65 kb promoter region of the *flhF* gene isolated from *P. putida* MK1 into the promoter proving vector pMK301. The promoter proving vector pMK301 was previously constructed, derived from a broad host range vector pMMB67EH combined with the *lacZ* as a reporter gene [8]. To efficiently clone such a small fragment as the 0.65 kb promoter region into a large plasmid, a two-step cloning strategy was applied. First, pMKU101 containing the promoter region was cut with *Hind*III, and then the 2.1 kb tetracycline-resistant gene digested from the pUT-mini *Tn5*::Tc was inserted, resulting in pMKT101. Second, the 2.75 kb DNA fragment containing the tetracycline-resistant gene and promoter region was digested with *EcoRI* and *Hind*III, and inserted into pMK301, generating pMKD101 (Fig. 1). Meanwhile, pMKT301 containing only the tetracycline-resistant gene was also constructed as a control. The plasmid pMKD101 was transformed into *P. putida* MK1 and preliminarily tested for the starvation promoter activity after growth on LB agar medium containing X-gal plus IPTG. On this preliminary test, the strain RPD101 (*P. putida* MK1 containg pMKD101) developed its color into
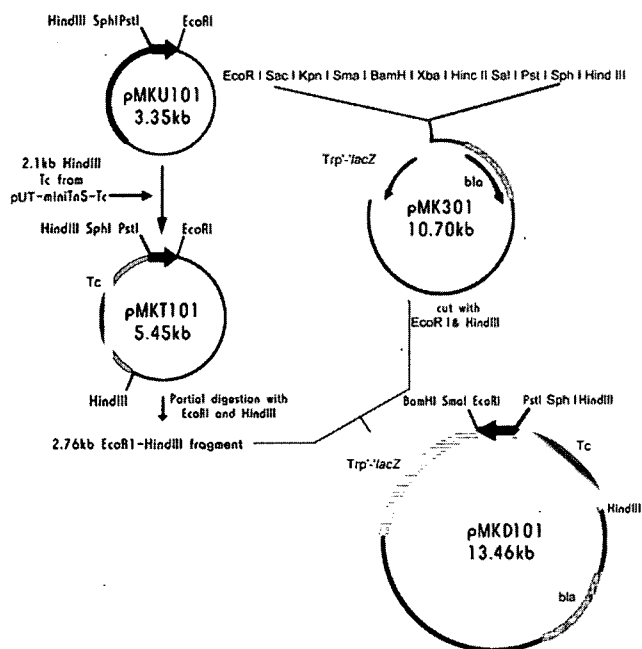
Fig. 1. Construction of plasmid pMKD101.

The pMKD101 was constructed by cloning the 0.65 kb promoter region of the *flhF* gene isolated from *P. putida* MK1 into the promoter proving vector pMK301. The promoter proving vector pMK301 was previously constructed, derived from a broad host range vector pMMB67EH combined with the *lacZ* as a reporter gene [8]. To efficiently clone such a small fragment as the 0.65 kb promoter region into the large plasmid, a two-step cloning strategy was applied.

dark blue in about 24 h of growth, when glucose was expected to be exhausted, indicating that the starvation promoter activity was active.

P. putida RPD101 was grown in 1 l of M9 medium plus 0.1% glucose in a fermentor under batch condition. Under this condition, the doubling time was about 4 h, and the exponential phase continued for about 9 h (Fig. 2). As expected, the strain expressed a low level of β-galactosidase activity during the exponential phase. The enzyme activity, however, increased up to 3.2 times higher than that of the exponential phase, when bacteria entered into the stationary phase.

In a chemostat culture, the growth rate equals the dilution rate. Tunner *et al.* [17] have reported that the starvation promoter-driven enzyme activity was significantly increased at the minimal dilution (growth) rate in a chemostat culture. To examine if this happened in our system, we investigated the β-galactosidase activities in different growth rates in a chemostat culture and compared the activity at both minimal and maximal growth rates. The composition of the medium in a chemostat culture was the same as that of batch culture with 0.1% glucose as a sole energy and carbon source. The maximal growth rate was obtained at a dilution rate of 0.173 h^{-1}. Under this condition, the cell mass, expressed as an O.D. value, was maintained

at about 0.13 after 10 h of incubation. The minimal growth rate was obtained at a dilution rate of 0.05 h^{-1} with an O.D. value of 0.35 after 7 h of incubation. These O.D. values of 0.13 and 0.35 would correspond to the values of the exponential and the stationary phases under batch culture condition, respectively. The β-galactosidase activity at the minimal growth rate in a chemostat was about 3 times higher than that at the maximal growth rate (Fig. 3). This result was similar to that obtained from the batch culture experiment, demonstrating that the promoter activity of a carbon starvation gene, *flhF*, is functional under the carbon-limited growth condition as well as under the carbon starvation condition. Meanwhile, the control strain, *P. putida* RPT301, expressed a low level of β-galactosidase activity both at the maximal and the minimal growth rates. Until the end of the chemostat experiments, the percentage of plasmid-bearing cells was over 98% and 96% in cultures maintained with maximal and minimal growth rates, respectively, indicating that plasmid stability at both cultures was very high.

In this study, we have demonstrated that a carbon starvation promoter can be utilized for the production of a foreign protein through recombinant DNA technology
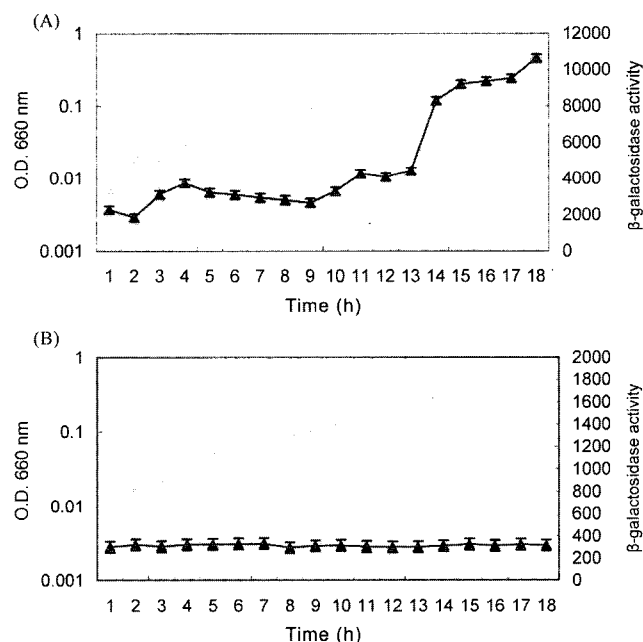
Fig. 2. Growth and β-galactosidase activity of RPD101 cultures (A) and RPT301 cultures (B).

The strain RPD101 containing the plasmid pMKD101 was grown on 1 l of M9 medium plus 0.1% glucose in a bioreactor under batch condition. Under this condition, the doubling time was about 4 h and the exponential phase was continued for about 9 h. As expected, the strain expressed a low level of β-galactosidase activity during the exponential phase. The enzyme activity increased up to 3.2 times higher than that of the exponential phase, when bacteria entered into the stationary phase. Symbols: ■, growth curve; ▲, β-galactosidase activity.

(A)

(B)
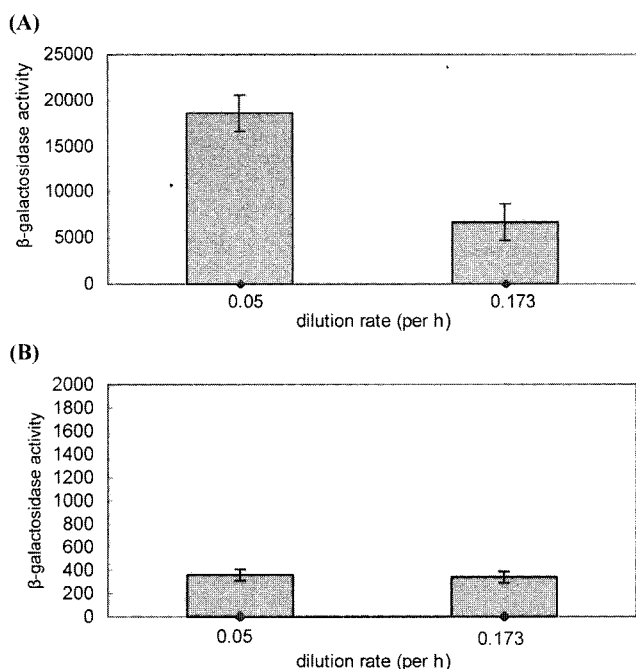
Fig. 3. β-Galactosidase activity during the chemostat culture of the strain RPD101.

The strain RPD101 with pMKD101 (A) and the control strain RPT301 (B) were continuously grown under the same condition as that of batch culture with 0.1% glucose as a sole energy and carbon source. β-Galactosidase activity was monitored at different dilution rates. Under the maximal growth condition, the cell mass, expressed as an O.D. value, was maintained at about 0.13. The minimal growth rate was obtained with an O.D. value of 0.35.

under nutrient limitation condition. We used the *flhF* promoter region with the *lacZ* as a reporter gene to control β-galactosidase activity under glucose-limited sluggish growth condition. Using this system, we were able to completely uncouple protein production from cell growth. Previously, Matin *et al.* [11] have also shown that the *cst1* system, one of the carbon starvation promoters, can be used to induce protein production in *E. coli* under growth-limited condition. The carbon starvation system used in this study is not from *E. coli*, but from *Pseudomonas* strain. *Pseudomonas* strain has the advantage over *E. coli* in some aspects: *Pseudomonas* strains are indigenous bacteria to natural environment and hence possess greater survivability than *E. coli* under carbon starvation condition, which bacteria often encounter in nature. *Pseudomonas* also expresses much stronger starvation promoter activity than that of *E. coli*, making this strain a suitable candidate for an efficient *in-situ* bioremediation. In the industrial fermentation process, it is desirable to concentrate microbial cells in bioreactors for rapid and efficient bioconversions. One of the technologies for the concentration of cells is the cell immobilization technique. Once cells are maximally immobilized onto a bioreactor, further growth of resident cells should be limited to prevent a bioreactor from

degeneration due to the overgrowth of the cell, which leads to a short life span of a bioreactor [6]. The carbon starvation promoter can be used effectively to remedy this drawback. Due to their long survivability and strong starvation promoter activities, *Pseudomonas* strains would efficiently perform their tasks as biocatalysts to express biochemical activity of interest at a high level during restricted growth in a bioreactor. Further investigation is now underway.

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