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Production of methionine γ-lyase in recombinant *Citrobacter freundii* bearing the hemoglobin gene

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The production of antileukemic enzyme methionine γ-lyase (MGL) in distinctly related bacteria, *Citrobacter freundii* and in their recombinants expressing the *Vitresocilla* hemoglobin (VHb) has been studied. This study concerns the potential of *Citrobacter freundii* expressing the *Vitreoscilla* hemoglobin gene (*vgb*) for the methionine γ-liyase production. Methionine γ-liyase production by *Citrobacter freundii* and its *vgb*$^-$ and *vgb*$^+$ bearing recombinant strain was studied in shake-flasks under 200 rpm agitation, culture medium and 30°C in a time-course manner. The *vgb*$^+$ and especially the carbon type had a dramatic effect on methionine γ-liyase production. The *vgb*$^+$ strain of *C. freundii* had about 2-fold and 3.1-fold higher levels of MGL than the host and *vgb*$^-$ strain, respectively. [BMB reports 2011; 44(9): 590-594]

INTRODUCTION

Enzymes far exceed man-made catalysts in their reaction specificity, catalytic efficiency, and capacity to operate under mild conditions of temperature and hydrogen ion concentration. As drugs, enzymes have two important features that distinguish them from all other types of drugs. First, enzymes often bind and act on their targets with great affinity and specificity. Second, enzymes are catalytic and convert multiple target molecules to desired products. These two features make enzymes specific and potent drugs that can accomplish therapeutic biochemistry in the body that small molecules cannot. These characteristics have resulted in the development of many enzyme drugs for a wide range of disorders (1). L-Methionine γ-lyase (MGL; EC 4.4.1.11) is a pyridoxal 5'-phosphate (PLP)-dependent multifunctional enzyme which catalyzes α, γ-elimination and γ-replacement of L-methionine and its derivatives and also α, β-elimination and β-replacement of S-substituted L-cysteines (2-4). This activity was first found using dried cells of *E. coli* and *Proteus vulgaris* (4). MGL have been isolated, purified, and characterized from several microorganisms such as *Pseudomonas putida*, *Clostridium sporogenes*, *Aeromonas* sp., *Citrobacter intermedius*, *Citrobacter freundii*, *Brevibacterium linens*, *Trichomonas vaginalis*, and *Porphyromonas gingivalis* (3-10).

MGL has not been found in yeast and mammals (7, 10, 11). Several practical applications of MGL have been reported. MGL has demonstrated antitumour efficacy in a number of methionine-dependent cancer cell lines including lung cancer, colon cancer, kidney cancer, brain cancer, prostate cancer, melanoma and fibrosarcoma (7). The antitumor efficacy of MGL, which is based on the greater L-methionine dependency of cancer cells for growth compared to normal cells has been investigated (3). Many human cancer cell lines and primary tumors have an absolute requirement for L-methionine, an essential amino acid (2). *In vitro* studies have demonstrated that several murine and human tumor cell lines need exogenous methionine to survive and proliferate (12). On the other hand, normal cells can utilize homocysteine to synthesis adequate levels of methionine and support a normal cell cycle. For these reasons; normal human cells are relatively resistant to exogenous L-methionine restriction (12). Numerous human cancer cell lines and primary tumors have an absolute requirement for L-methionine, an essential amino acid. On the other hand, normal human cells are relatively resistant to exogenous L-methionine restriction. Thus, depletion of L-methionine is effective for cancer therapy (13). Upon L-methionine depletion, L-methionine- dependent cancer cells are not able to divide and become arrested in the late-S/G_2 phase of the cell cycle (2, 3, 12, 14). Cell cycle arrested and death can be reversed by addition of exogenous methionine (12). On the other hand, normal human cells are relatively resistant to exogenous L-methionine restriction. Thus, depletion of L-methionine is effective for cancer therapy. The therapeutically utility of the enzyme for depleting L-methionine has been reported (2). The enzyme has been found to be an effective anti-tumor agent *in vitro* and *in vivo* and to be of potential value in the treatment of Parkinson's disease, arteriosclerosis, aging and obesity (9).

Citrobacter freundii, Gram-negative, nonperforming rods belonging to the family *Enterobacteriaceae* and commonly found in soil, water, sewage, food, and the intestinal tracts of animal and human (15, 16).

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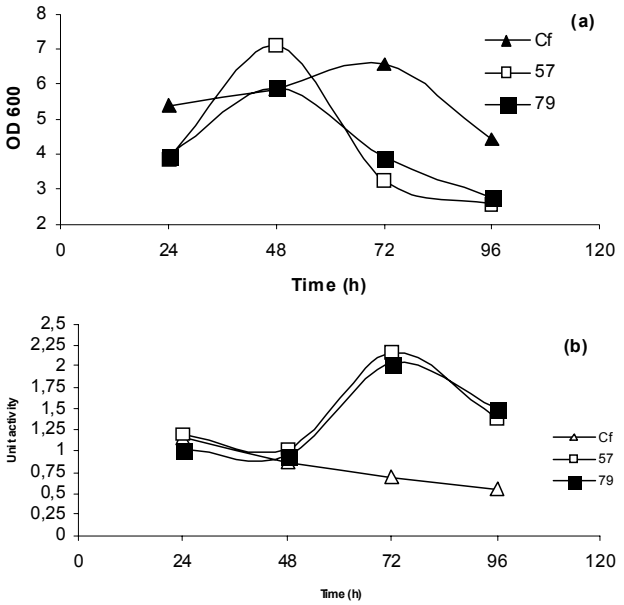

Fig. 1. Total cell mass (a) and (b) intracellular MGL levels of *C. freundii* and their MK57 and MK79 harboring recombinants grown in LB medium for 24-96 h. Cells were grown a gyratory water-bath at 30°C in 150-ml volume flasks containing 50 ml culture medium. Each value is the average of at least three independent experiments. For clarity, no error bars are given, but they are mostly less than 10% of the respective data point.

In the aerobic bacterium *Vitreoscilla*, the synthesis of homodimeric hemoglobin (VHb), currently the only well known prokaryotic hemoglobin, can be induced under hypoxic conditions. The expression is maximally activated both in *Vitreoscilla* and *E. coli* under micro aerobic conditions, when the dissolved oxygen levels drops below 10% of air saturation. The *vgb* has been cloned into a variety of bacteria and eukaryotic organism mostly for the purpose of enhancing respiration, growth and productivity (17).

The aim of the present study was to determine the potential of VHb, protein known for its beneficiary effect on cell growth on metabolite production, on MGL production, an anticancer agent *C. freundii*. The presence of *vgb*/VHb may prove useful in this application.

RESULTS AND DISCUSSION

The production of MGL was studied in *C. freundii* and in their recombinants harboring the *vgb*. The effect of VHb on production of MGL by *C. freundii* was distinct. The strain of this bacterium, MK79, expressing VHb responded differently to carbon catabolite repression of the enzyme. The intracellular MGL level was determined cultures harvested at 96 h. Our preliminary studies showed that this compound was produced during the late (post-stationary) secondary phase of growth (Fig. 1b,

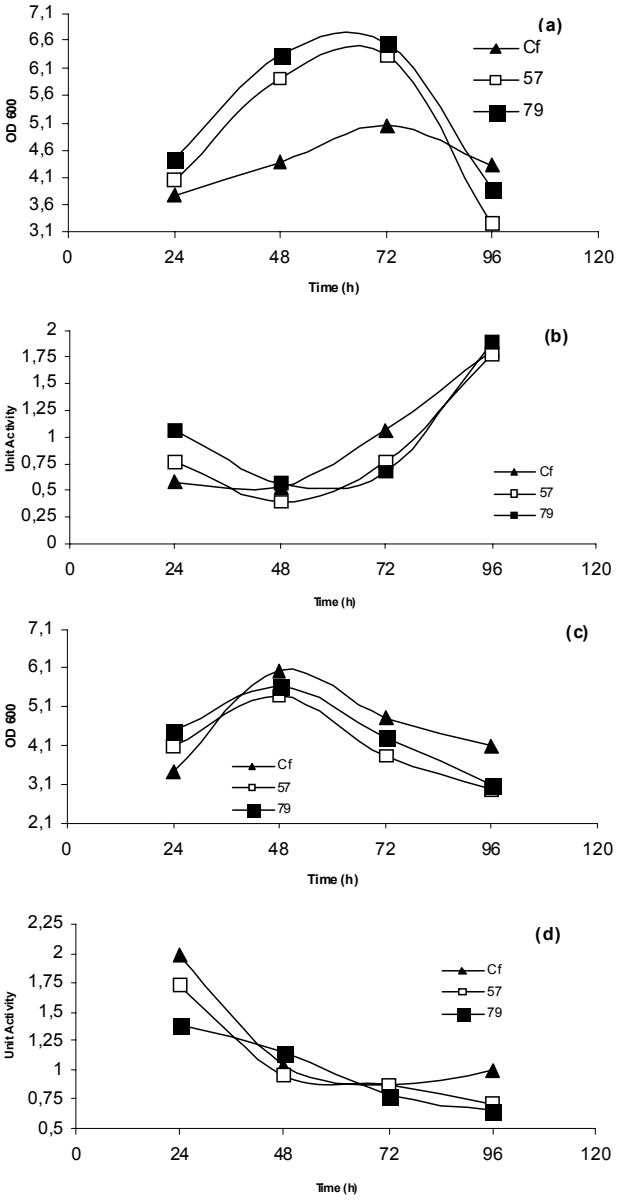

Fig. 2. Total cell mass (a, c) and (b, d) intracellular MGL levels of *C. freundii* and their h MK57 and MK79 harboring recombinants grown in % 1 (a, b) 0.1% glucose (c, d) supplemented LB medium for 24-96 h. Cells were grown a gyratory water-bath at 30°C in 150-ml volume flasks containing 50 ml culture medium. Each value is the average of at least three independent experiments. For clarity, no error bars are given, but they are mostly less than 10% of the respective data point.

2b). On further incubation (e.g., 72 h and up), a substantial decrease in MGL production was observed. The effect of various conditions on the production of MGL, the enzyme converting L-methionine to its derivate, was also investigated.

In LB-grown cultures, the intracellular MGL level of recombinant MK79 strains was substantially higher than that of their counterparts, the *vgb*$^{-}$ and wild type strains (Fig. 1b, 2b, 3d).

In MM-grown cultures, the intracellular MGL level of wild-type strains of *C. freundii* was substantially higher than that of their respective recombinants, the *C. freundii*, MK57 and MK79 strains (Fig. 2b, 2d). Although to a lesser extent, the intracellular levels of MGL for the wild-type strain of were also higher than for their recombinant counterparts. In *C. freundii* and its recombinants, there was inverse relationship between the total cell mass and intracellular product level; the highest product formation was observed in cultures with lowest total cell mass (Fig. 3b, d). The lower levels of intracellular MGL in recombinant bacteria (*C. freundii*, MK57 and MK79) may be due to plasmid burden on cells (Fig. 2b, 2d, 3b).

The effect of plasmid-mediated metabolic burden on the expression of the host genes has been the subject of some studies, in which biosynthetic burden associated with plasmid presence was shown to limit the cell growth, the metabolites studied and the recombinant protein production (18). Thus, in the current study, the burden that the presence of plasmid exerts on the cells may well exceed the beneficial effect of VHb. Lower intracellular MGL levels of *C. freundii*, MK57 and MK79 recombinants of bacteria may also be due to a higher metabolic burden inflicted by the presence of these high copy-number plasmids. The MK79 strain of *C. freundii* had about 2-fold and 3.1-fold higher levels of MGL than the host and *C. freundii*, MK57 strains, respectively (Fig. 1b, 2b). The MK79 strain of *C. freundii* had about 2-fold higher total cell mass levels than MK57 strain. This finding are in agreement with those from previous studies showing that the dramatic effect of VHb on cell metabolism and production formation is found under limited oxygen conditions where an elevated expression of VHb occurs (19). Industrial biotechnology is becoming increasingly important as the unique metabolic capacities of microbes offer new synthetic routes for a broad range of valuable products from fuels and materials to fine chemicals (20). MGL is one of a few microbial enzymes with high therapeutic value in certain kinds of cancers, where it is used to "starve" cancer cells of amino acid L-methionine. The chemotoxic effect of the enzyme is a result of such restriction, which causes growth inhibition of cancer cells. This enzyme does not occur naturally in humans, but is found in bacteria. The enzyme with chemotherapeutic potential is, however, only present in several gram-negative bacteria. The utilization of this recombinant oxygen uptake system (VHb) in an aerobic fermentation process (MGL production) may be advantageous. With the use of this system in bioprocesses, oxygen can be buffered at low concentrations and its transfer rate can be increased in aging cells. Oxygen deficiency that develops in the fermentation with a low oxygen transfer rate has significant effects, such as low viable cell number which results low product yield. The purpose of this study was to determine whether an efficient oxygen-uptake system, the VHb, provides an advant-

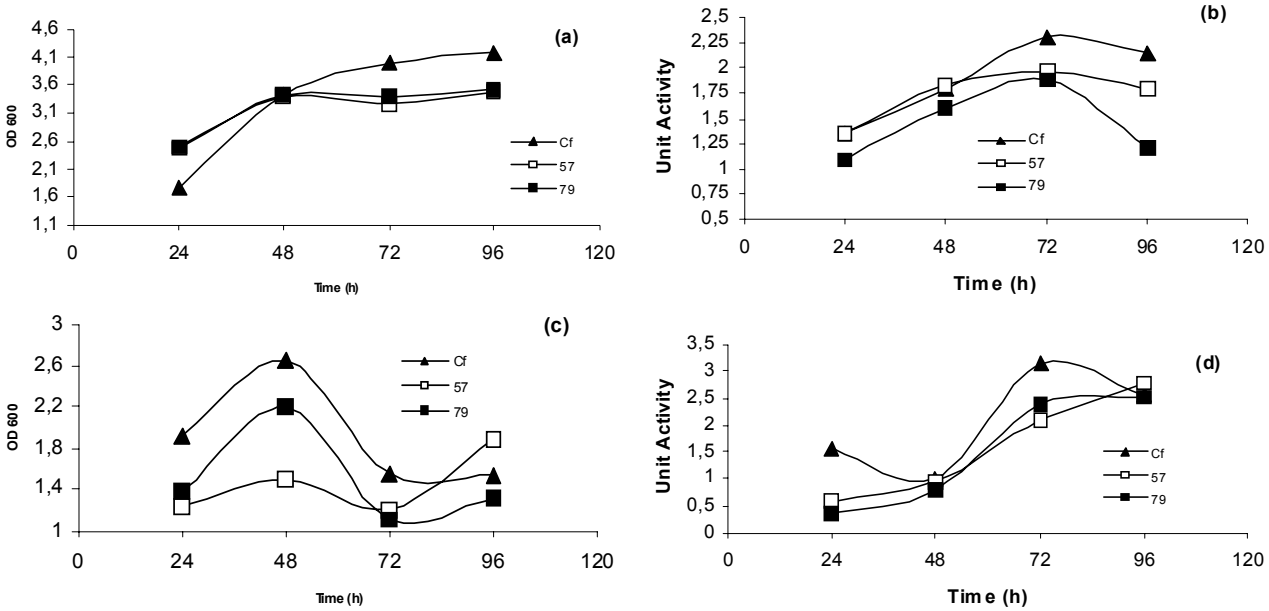

Fig. 3. Total cell mass (a, c) and (b, d) intracellular MGL levels of *C. freundii* and their h MK57 and MK79 harboring recombinants grown in % 1 (a, b) 0.1% glucose (c, d) supplemented MM medium for 24-96 h. Cells were grown a gyratory water-bath at 30°C in 150 ml volume flasks containing 50 ml culture medium. Each value is the average of at least three independent experiments. For clarity, no error bars are given, but they are mostly less than 10% of the respective data point.

age for production of MGL, in this enzyme with high importance in health industry. The MK79 strain has substantially higher MGL activity than the respective wild-type host or the strain bearing a comparable plasmid without *vgb*.

MATERIALS AND METHODS

Chemicals and reagents

Methionine (Aldrich); PLP, mercaptoethanol (Sigma), EDTA (Applichem), TCA (Across), 3-MBTH (Merck); sodium acetate (Riedel-de Haen), potassium phosphate and di-potassium phosphate (Sigma). All other chemicals were of analytical grade.

Bacterial strain and culture conditions

The bacterial strain used in this study was *Citrobacter freundii* (NRRL B-2643) and their *vgb*$^{+}$ and MK57 recombinant strain. *C. freundii* was transformed with pMK57 (Fig. 4a) and its vgb carrying recombinant plasmid pMK79 (Fig. 4b), the cells were maintained on LB agar plates at 4ºC with transfers at monthly intervals. The liquid media used throughout the study were Luria-Bertani (LB) broth and minimal medium (M9 Minimal media).

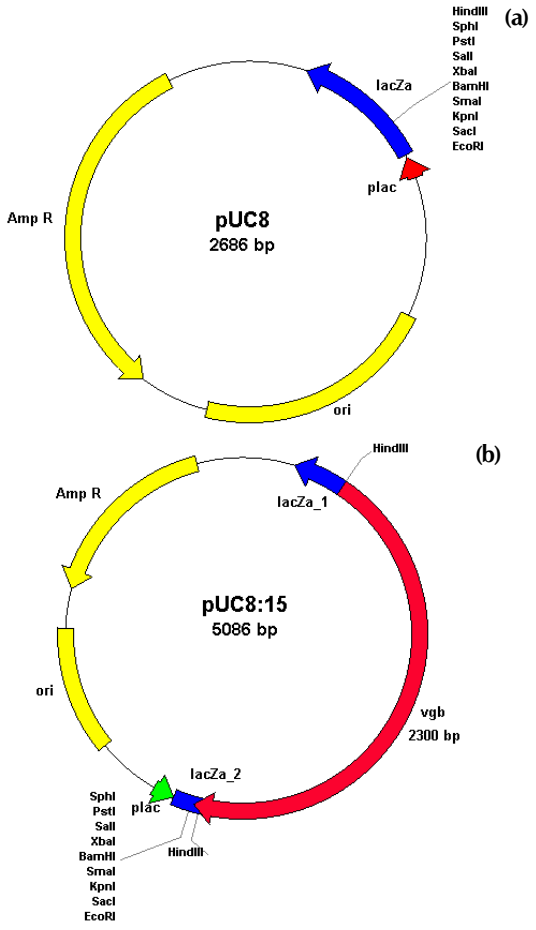

Fig. 4 (a) Genetic map of plasmid "pMK57". (b) Genetic map of plasmid "pMK79". Amy, α-amilase gene.

Growth conditions

Cells were maintained on Luria-Bertani (LB) (Miller, 1972) agar plates at 4°C with transfers at monthly intervals. A 1/100 inoculum of overnight cultures grown in LB was made in 50 ml LB in 150 ml Erlenmeyer flasks. Inocula in flasks were grown for 24 h at 30°C in a 200 rpm water-bath. *C. freundii* strains were routinely grown on Luria-Bertani medium (LB). Minimal medium contained 1% and 0.1% glucose, added from a stock solution respectively, autoclaved. LBG medium contained 1% and 0.1% glucose. The growth characteristics of both wild *C. freundii* and its recombinants in the rich (LB) and in the nutrient restricted nutritional (MM) media were monitored at the given intervals during the course of the experiments.

For MGL production and assay

For MGL production, 250 µl of this O/N culture was then inoculated into 150 ml conical flask containing 50 ml of sterile mineral salts medium supplemented with 1% or 0.1% (w/v) glucose, respectively. This was incubated for 96 h at 30°C, 200 rpm on an orbital shaker. Recombinant MGL activity was determined by the method described (21, 22). The absorbance measured at 320 nm a unit's enzyme. One unit will release 1.0 micromole of alpha-ketobutyrate from L-methionine per minute at pH 8.0 at 37°C.

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