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Characterization of *Methylophaga* sp. strain SK1 Cytochrome c_L Expressed in *Escherichia coli*

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***Methylophaga* sp. strain SK1 is a new restricted facultative methanol-oxidizing bacterium that was isolated from seawater. The aim of this study was to characterize the electron carriers involved in the methanol oxidation process in *Methylophaga* sp. strain SK1. The gene encoding cytochrome c_L (*mxaG*) was cloned and the recombinant gene was expressed in *Escherichia coli* DH5α under strict anaerobic conditions. The recombinant cytochrome c_L had the same molecular weight and absorption spectra as the wild-type cytochrome c_L both in the reduced and oxidized forms. The electron flow rate from methanol dehydrogenase (MDH) to the recombinant cytochrome c_L was similar to that from MDH to the wild-type cytochrome c_L. These results suggest that recombinant cytochrome c_L acts as a physiological primary electron acceptor for MDH.**

Key words: cytochrome c_L, methanol dehydrogenase (MDH), methanol oxidation, *Methylophaga* sp. strain SK1

Cytochrome *c* is an electron transport protein that is involved in the respiratory processes of almost all organisms. The main distinguishing feature of this protein family is the presence of a heme group covalently linked to a polypeptide by one or two thioether bonds (Moore and Pettigrew, 1990). In Gram-negative soil methylotrophic bacteria, methanol is oxidized to formaldehyde by periplasmic methanol dehydrogenase (MDH), which has a pyrroloquinoline quinone (PQQ) as a prosthetic group and cytochrome c_L as its primary electron acceptor (Anthony, 1986). MDH and cytochrome c_L are found in the periplasm at high concentrations and form the first part of a methanol oxidation electron transport chain. A typical class I type cytochrome (cytochrome c_H) is also involved in methanol oxidation, and cytochrome c_H is oxidized by a terminal oxidase according to the following scheme:

Methanol→MDH→cyt. c_L→cyt. c_H→terminal oxidase

Most soil methylotrophic bacteria contain at least two soluble *c*-type cytochromes whose molecular weights, isoelectric points and absorption spectra differ from each other. The *c*-type cytochromes can be classified based on the isoelectric point and the isoelectric point of cytochrome c_L is usually lower than that of cytochrome c_H. A new restricted facultative marine methanol-oxidizing bacterium, *Methylophaga* sp. strain SK1, was isolated from the seawater at Mokpo, Korea, and the electron carriers involved in the process of methanol oxidation in this bacterium were characterized (Koh *et al.*, 2002).

There are several reports regarding the expression of bacterial *c*-type cytochromes, which address both homologous and heterologous strategies (Karan *et al.*, 2002; Kellogg and Bren, 2002; Londer *et al.*, 2002; Miller *et al.*, 2003; Evers and Merk, 2005). This study describes the cloning and expression of the gene, *mxaG*, which encodes cytochrome c_L from *Methylophaga* sp. strain SK1. Under strict anaerobic conditions, the heterologous expression of the *mxaG* gene can be achieved in *E. coli* DH5α at levels that allow the protein to be isolated. The heterologously expressed protein was purified and compared with the cytochrome c_L isolated from the *Methylophaga* sp. strain SK1.

Materials and Methods

Bacteria, media and culture conditions

The *Methylophaga* sp. strain SK1 was grown aerobically in an artificial seawater medium (NaCl 30 g, NaH_2PO_4 2.34 g, K_2HPO_4 6.1 g, $(NH_4)_2SO_4$ 1.0 g, $NaNO_3$ 1.0 g, $MgSO_4 \cdot 7H_2O$ 0.2 g, $CaCl_2$ 0.01 g, and 1 ml of trace elements solution per liter) containing 1% (v/v) methanol as the sole carbon and energy source. Expression studies were carried out using *E. coli* DH5α, which were performed using rich TYS medium (1% trypton, 1% yeast extract,

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0.5% NaCl and 50 μg/ml ampicillin) at 37°C.

Construction of genomic library and identification of mxa genes

DNA manipulation was performed using the method reported by Sambrook and Russell (2001). A genomic library of the *Methylophaga* sp. strain SK1 was constructed by cloning the *Hin*dIII treated chromosomal DNA fragments into the pUC19 vector. A 5.4 kb *Hin*dIII fragment of genomic DNA was selected using colony hybridization with the conserved region of *mxaF* as a probe, which encodes the α-subunit of methanol dehydrogenase involved in the methanol oxidation. The fully sequenced fragment revealed four tightly linked genes (encoding partial *mxaF*, *mxaJ*, *mxaG*, and *mxaI*) to be involved in methanol oxidation.

Construction of expression plasmids

The Plasmid pGEM T-easy (Promega, USA) was first ligated with the *mxaG* gene. The *mxaG* gene was subcloned from the genomic library using the following primer set (5'-CTCGAGTTATAAAAGTGGGATGCC-3' and 5'-AAACTCGAGTCAAATAGGCTTAGATCT-3'. The underline sequences indicate the *Xho*I and *Xba*I sites, respectively). The resulting plasmid was digested using *Xho*I and *Xba*I, and the DNA fragment containing *mxaG* was ligated into the expression vector, pASK-IBA6, that had been digested with *Xho*I and *Xba*I. The recombinant plasmid pEC05 was introduced into the *E. coli* DH5α cells by electroporation. An *E. coli* DH5α was transformed using this vector and incubated at 37°C overnight on an LB agar plate containing ampicillin. The recombinant plasmid contained the 585 bp DNA fragment originating from *Methylophaga* sp. strain SK1.

Expression and purification of recombinant cytochrome c_L from E. coli

Fresh colonies of *E. coli* DH5α containing the pEC05 plasmid were inoculated into the TYS medium and grown overnight under aerobic conditions. The preculture was inoculated into the TYS medium fully contained in a 5 L flask under strict anaerobic conditions, which were maintained by continuous flushing the growth chamber with N_2. The growth medium was supplemented with 50 μg/ml ampicillin, 200 ng/l tetracycline and 10 mM KNO_3, and *mxaG* gene expression was induced with 0.1 mM IPTG when the OD_{600} reached 0.5 - 0.6. The cells were harvested by centrifugation at 15,000 × *g* for 5 min at 4°C. The pellet was resuspended in 10 ml of a precooled buffer P (500 mM sucrose, 1 mM EDTA, 10 mM Tris-HCl, pH 8.0) containing 1 mg/ml lysozyme and placed in ice for 30 min. The suspension was then centrifuged at 20,000 × *g* for 5 min at 4°C. The clear supernatant was applied to a Ni-NTA affinity column in order to purify the recombinant protein with 6×His-tagged proteins using the QIA express system. Heme staining was carried out according to the method reported by Thomas *et al.* (1976).

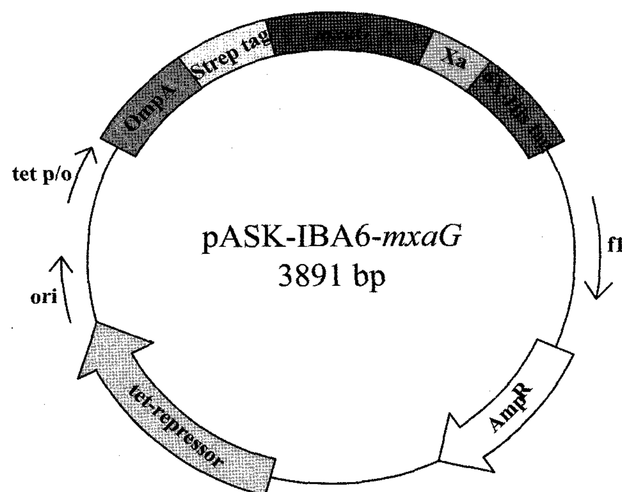

Fig. 1. Construction of the pEC05 vector.

Electron flow from MDH to cytochrome c_L

The electron flow from MDH to cytochrome c_L was determined using potassium ferricyanide as the final electron acceptor. The assay mixture contained 3 μM $K_3Fe(CN)_6$, 13.4 mM MeOH, and 25 μM cytochrome c_L. The reaction was started by adding MDH (20 μM). The rate of potassium ferricyanide reduction was determined by measuring the decrease in absorbance at 420 nm. The rate of electron flow from the MDH to cytochrome c_L was also determined using the following procedure: cytochrome c_L was oxidized completely by adding potassium ferricyanide, and the excess reagent was removed by gel filtration using a PD-10 column. Subsequently, active MDH (20 μM) was added to the cytochrome c_L solution, and the rate of cytochrome c_L reduction was determined by measuring the increase in absorbance at 551 nm.

Results and Discussion

Expression and purification of recombinant cytochrome c_L

The heterologous expression of a bacterial *c*-type cytochrome in *E. coli* has been problematic because *E. coli* lack the ability to covalently attach the cysteine thiols to protoporphyrin IX, which has been shown to be a crucial step for yeast to produce the enzyme heme *c* lyase (Dumont *et al.*, 1987). An analogous enzyme has not been identified in *E. coli* but *E. coli* produces an assimilatory nitrite reductase containing heme *c* under anaerobic conditions. Similarly, mature *Thiobacillus versutus* cytochrome *c*-550 (Ubbink *et al.*, 1992), *Chromatium vinosum* cytochrome *c*' (Even *et al.*, 1995), cytochrome *c*-551 (Miller *et al.*, 2000), horse cytochrome *c* (Kellogg and Bren, 2002) and equine cytochrome *c* (Rumbley *et al.*, 2002) have been successfully expressed in *E. coli* at rea-

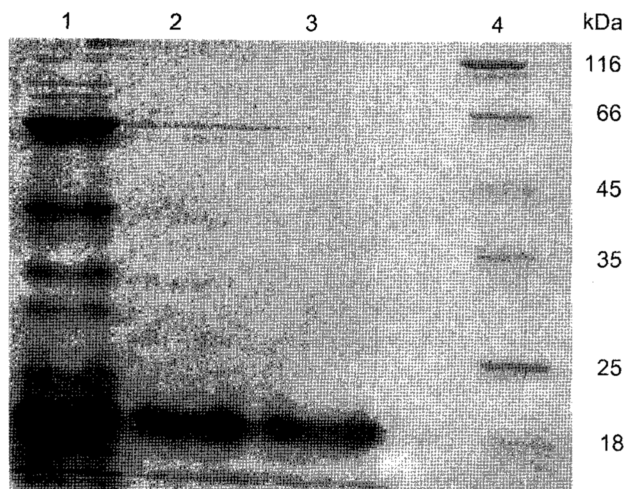

Fig. 2. SDS-PAGE analysis of the wild-type and recombinant cytochrome c_L. Lane 1, cell-free extract; lane 2, purified wild-type cytochrome c_L; lane 3, purified recombinant cytochrome c_L; lane 4, protein size markers.

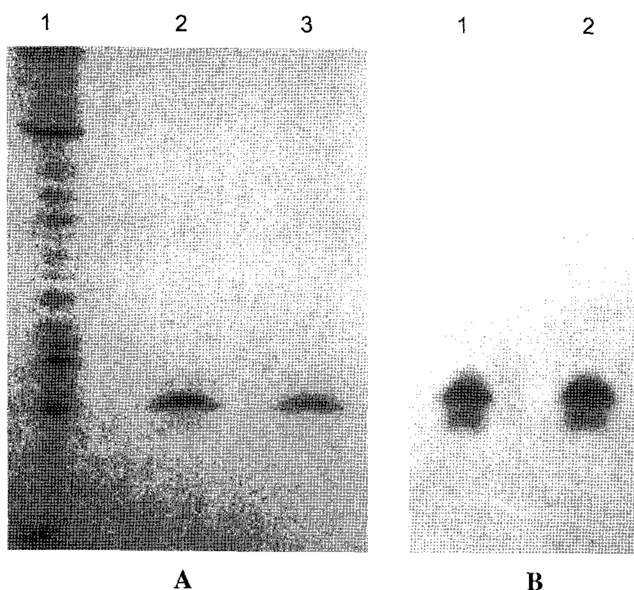

Fig. 3. A, Native PAGE analysis of the wild-type and recombinant cytochrome c_L. Lane 1, cell-free extract; lane 2, purified wild-type cytochrome c_L; lane 3, purified recombinant cytochrome c_L. B, Heme staining of cytochrome c_L. Lane 1, wild-type cytochrome c_L; lane 2, recombinant cytochrome c_L.

sonable levels.

E. coli DH5α containing the *mxaG* gene was used to produce preparative amounts of the His-tagged protein. Recombinant cytochrome c_L containing the 6×His-tagged proteins was bound to Ni-NTA resin. The bound protein was washed with 250 mM Tris-HCl buffer containing 10 mM imidazole and 300 mM NaCl and eluted with a linear gradient of 20 - 250 mM imidazole in the same buffer. Fractions containing the respective proteins were

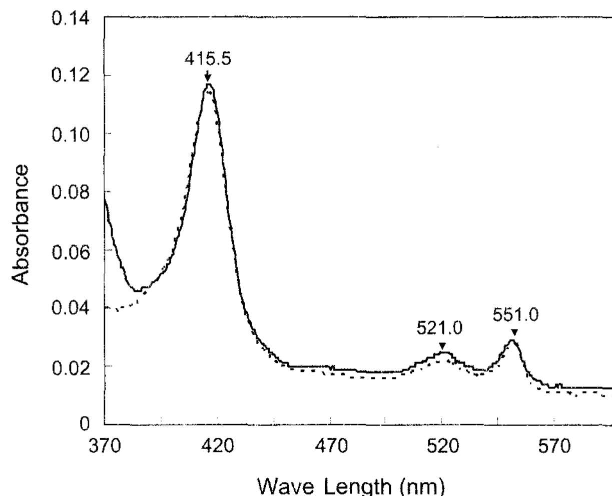

Fig. 4. Comparison of reduced absorption spectra of the wild-type cytochrome c_L (—) and recombinant cytochrome c_L (····).

pooled and concentrated. Yields of the recombinant cytochrome c_L obtained were approximately 0.3 mg/l of culture. Fig. 2 shows the SDS-PAGE analysis of the authentic and recombinant cytochrome c_L. The molecular weight of the recombinant cytochrome c_L was similar to that of the wild-type cytochrome c_L (21 kDa). Fig. 3A and B show the native PAGE and heme staining of the authentic and recombinant cytochrome c_L, respectively. According to the molecular weight measurement and heme staining results, the heme was covalently linked to the recombinant protein. Fig. 4 shows the absorption spectra of the reduced wild-type and recombinant cytochrome c_L. Both proteins had the same three absorption peaks at 551.0, 521.0 and 415.5 nm.

Electron flow from MDH to cytochrome c_L

Fig. 5A shows the electron flow rates from MDH to the wild-type or recombinant cytochrome c_L, which had been oxidized by potassium ferricyanide prior to the reaction. The oxidized cytochrome c_L was reduced by MDH and the absorbance at 551.0 nm increased in a time-dependent manner. The electron transfer reaction was complete within 20 sec.

Electron flow between the electron carriers during methanol oxidation was determined using an artificial electron transport system in which potassium ferricyanide was used as the final electron acceptor (Koh *et al.*, 2002). The decrease in absorbance at 420 nm as a result of the reduction of potassium ferricyanide was monitored. Fig. 5B shows the rates of electron transfer from MDH to potassium ferricyanide via cytochrome c_L. Regardless of the source of cytochrome c_L, the potassium ferricyanide was completely reduced within 20 min.

In summary, a recombinant cytochrome c_L from *Methylophaga* sp. strain SK1 was expressed in *E. coli* DH5α and characterized under strict anaerobic conditions. The

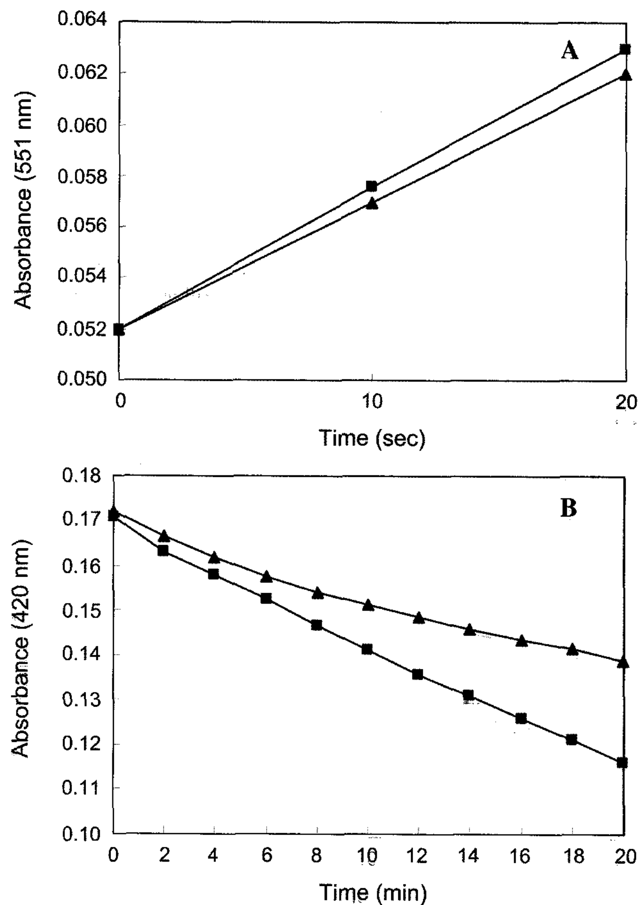

Fig. 5. A, Comparison of the reduction rates of the wild-type and recombinant cytochrome c_L. The wild-type and recombinant cytochrome c_L (25 μM) were oxidized with 3 μM potassium ferricyanide and the oxidant was removed by passage through a PD-10 column. The reaction rates were measured by the increase in absorbance at 551.0 nm. Each reaction mixture contained either the oxidized wild-type cytochrome c_L (▲) or recombinant cytochrome c_L (■), and the reactions were started by adding MDH (20 μM). B, Reduction of potassium ferricyanide with MDH and cytochrome c_L (wild-type or recombinant type). The reduction rates were measured by the decrease in absorbance at 420 nm at room temperature. The assay systems differed from each other as follows: (▲), 3 μM $K_3Fe(CN)_6$, 13.4 mM MeOH, 25 μM wild-type cytochrome c_L; (■), 3 μM $K_3Fe(CN)_6$, 13.4 mM MeOH, 25 μM recombinant cytochrome c_L. The reactions were started by adding MDH (20 μM).

biochemical and spectroscopic properties of the recombinant cytochrome c_L were the same as those of the wild-type. This indicates that both the wild-type and recombinant cytochrome c_L act as physiological primary electron acceptors for MDH and will be useful for examining the roles of the *mxaG* gene in the *mxaFJGIR* gene cluster of *Methylophaga* sp. strain SK1.

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