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Purification and Some Properties of Soluble Cytochrome *c* from *Methylobacillus* sp. Strain SK1

Kim, Si Wouk, Young Tae Ro[1] and Young Min Kim[1]*

Department of Environmental Science, College of Natural Science, Chosun University, Kwangju 501-759
*[1]*Department of Biology, College of Science, Yonsei University, Seoul 120-749, Korea*

Methylobacillus sp. Strain SK1에 존재하는 Soluble cytochrome *c*의 정제 및 특성

김시욱 · 노영태[1] · 김영민[1]*

조선대학교 자연과학대학 환경학과
[1]연세대학교 이과대학 생물학과

ABSTRACT: Three types of soluble cytochrome *c* were purified to homogeneity from *Methylobacillus* sp. strain SK1 which grows only on methanol. Cytochrome *c*-I was purified 58.5-fold in seven steps. Cytochrome *c*-II and *c*-III were purified 57.3- and 122.1-fold in eight steps, respectively. The molecular weights of the cytochrome *c*-I was determined to be 12,500, while those of the cytochrome *c*-II and *c*-III were 16,000. The isoelectric points of the *c*-I, *c*-II, and *c*-III were found to be 8.8, 6.6, and 6.6, respectively. The spectrum of reduced cytochrome *c*-I showed α-, β- , γ-peaks at 551.4, 522.2, and 416.6 nm. The peaks for *c*-II were found at 551.0, 521.6, and 416.5 nm, while those for *c*-III were shown at 551.2, 521.8, and 416.0 nm. The spectra of oxidized cytochrome *c*-I, *c*-II, and *c*-III showed γ-peak at 411.8, 409.0, and 410.2 nm, respectively. The absorption coefficients of α-and γ- peak for *c*-I in the reduced state were determined as 47 and 197 $mM^{-1}cm^{-1}$, respectively. The coefficients of α- and γ-peak for *c*-II were determined to be 43 and 137 $mM^{-1}cm^{-1}$, while those for *c*-III were 41 and 172 $mM^{-1}cm^{-1}$, respectively. The *c*-I and *c*-III were found to bind carbon monoxide.

KEY WORDS □ Cytochrome *c*, Methylotroph, *Methylobacillus* sp. SK1, Methanol, Methanolotroph

INTRODUCTION

Methylotrophic bacteria are a group of bacteria that can grow aerobically on compounds containing one or more carbon atoms but no carbon to carbon bonds (Anthony, 1982). The methanol dehydrogenase (MeDH) in methylotrophic bacteria donates electrons obtained from methanol to the respiratory chain at the level of *c*-type cytochromes (Dijkstra *et al.*, 1989). These cytochromes are thought to mediate electron flow from the enzyme, in the order of MeDH-cytochrome c_L-cytochrome c_H-terminal oxidase (Anthony, 1986).

Since Anthony (1975) first studied the cytochromes from *Methylobacterium extorquens* AM1 (previously *Pseudomonas* AM1), several kinds of *c*-type cytochromes were purified from methylotrophic bacteria. Two or three soluble *c*-type cytochromes with different chemical and physical properties were reported to present in several obligate and facultative methylotrophic bacteria such as *Methylobacterium extorquens* AM1 (Day *et al.*, 1990; O'Keeffe and Anthony, 1980), *Methylophilus methylotrophus* (Cross and Anthony, 1980; Santos and Turner, 1988), *Hyphomicrobium* X (Dijkstra *et al.*, 1988), *Methylomonas* J (Ohta and Tobari, 1981), and *Methylomonas* sp. YK 56 (Tani *et al.*, 1986). *Paracoccus denitrificans*, an autotrophic methanol utilizer, also was found to have three

* Corresponding author.

soluble *c*-type cytochromes in the periplasm (Husain and Davidson, 1986). Bosma *et al.* (1987) and Long and Anthony (1991), however, suggested the presence of a new diheme cytochrome *c* and a novel heterodimeric cytochrome *c*, respectively, in *P. denitrificans*.

Methylobacillus sp. strain SK1 is a new obligate methanol-oxidizing bacterium (methanolotroph) which grows only on methanol (Kim *et al.*, 1991). We report in this paper the molecular and physical properties of soluble *c*-type cytochromes purified from *Methylobacillus* sp. strain SK1 to understand more about the mechanism of methanol oxidation in methylotrophic bacteria.

MATERIALS AND METHODS

Bacterial strain and Cultivation

Methylobacillus sp. strain SK1 was cultivated at 30℃ in a modified mineral medium of Kim and Hegeman (1981) supplemented with 1.0% (v/v) methanol; the pH and phosphate concentration of the medium were 7.5 and 50 mM, respectively, and the medium contained an additional mineral salt $NaNO_3$ (1.0 g/*l*) and a high concentration of $CuSO_4$ (1.0 mg/*l*).

Purification of soluble cytochrome *c*

All purification steps were carried out at 4℃. Cytochrome *c* was detected spectrophotometrically by the method of Tani *et al.* (1985). Cell pastes prepared from cells growing at the early stationary growth phase were resuspended in 10 mM Tris-HCl buffer (pH 8.0, standard buffer) and were disrupted by sonic treatment. The suspension was centrifuged at 15,000×*g* for 30 min. The resulting supernatant was then centrifuged at 100,000×*g* for 90 min. The supernatant was referred to as soluble fraction. Solid ammonium sulfate was added to the soluble fraction to give 35% saturation. After 30 min, the fraction was centrifuged at 15,000×*g* for 30 min. Ammonium sulfate was further added to the resulting supernatant to bring a final concentration of 90% of saturation. After 3 h, the solution was centrifuged at 15,000×*g* for 30 min. The precipitate was suspended in a small volume of standard buffer and dialyzed against two 10-liter changes of the same buffer for 24 h. The dialyzed solution was applied to a DEAE-Sephacel column (2.6×12.5 cm) which had been pre-equilibrated with the same buffer. A small amount of cytochrome *c* which was not bound to the column came straight through the column (designated cytochrome *c*-I), and the remainder was left bound to the column. The bound cytochrome *c* was eluted with standard buffer, using a linear gradient of 0-0.5 M NaCl.

The cytochrome *c*-I fractions were pooled and concentrated in an Amicon concentrator with an ultrafiltration membrane (YM-10) under an atmosphere of N_2 gas. The concentrates were dialyzed against 10 mM sodium phosphate buffer (pH 6.0), and applied to a CM-cellulose column (4×11 cm) which had been pre-equilibrated with the same buffer. The cytochrome *c*-I bound at the top of the column was eluted with a linear gradient of 10-100 mM sodium phosphate buffer (pH 6.0). The eluate was concentrated and applied to a Sephadex G-150 column (1.8×90 cm) which had been equilibrated with 25 mM Tris-HCl buffer (pH 8.0) and eluted with the same buffer. The cytochrome *c*-I fraction was further purified on a Sephadex G-75 column (2.4×18 cm) by elution with the same buffer.

The eluate of the bound cytochrome *c* from DEAE-Sephacel column was pooled, concentrated, and applied to a CM-cellulose column (4×11 cm) pre-equilibrated with 10 mM sodium phosphate buffer (pH 6.0). The cytochrome *c* fractions came straight through the column and were separated into two types. One was in the reduced form and the other was in the oxidized form. They were designated cytochrome *c*-II and *c*-III, respectively. The latter fraction was green in color. Each fraction was concentrated by ultrafiltration through YM-10 membrane before gel filtration on a Sephadex G-150 column (1.8×90 cm) using 25 mM Tris-HCl buffer (pH 8.0). After gel filtration, cytochrome *c*-II and *c*-III fractions were re-applied to the CM-cellulose column (4×11 cm) pre-equilibrated with 10 mM sodium phosphate buffer (pH 6.0). Each unbound cytochrome *c* fraction was concentrated by ultrafiltration using YM-10 membrane. Each concentrate was then applied to a Sephadex G-75 column (2.4×18 cm) and eluted with 25 mM Tris-HCl (pH 8.0).

The purified *c*-I, *c*-II and *c*-III were dialyzed extensively against distilled water.

Molecular weight determination

The molecular weights of purified cytochromes were determined using a Pharmacia FPLC System equipped with a Superose 12 column (1×30 cm). Elution of cytochromes and reference proteins was carried out at a rate of 0.5 m*l* per min with 0.1 M potassium phosphate buffer (pH 6.8) containing 0.1 M NaCl.

Absorption spectra determination

Spectrophotometric analysis of the purified cytochromes was performed with a Shimadzu UV-260 spectrophotometer using 10 mm light path cuvettes at room temperature. The absorption spectra of reduced or oxidized forms were measured after addition of dithionite or ferricyanide, respectively, to the purified cytochrome *c* solution. The amount of cytochrome *c* was calculated from the difference between the absorptions measured at 551.5 and 535 nm by the method of Tani *et al.* (1985). To detect the binding of cytochrome with carbon monoxide (CO), each cytochrome solution in two cuvettes which had been flushed

Table 1. *Purification of soluble cytochrome c-I, c-II, and c-III from Methylobacillus sp. strain SK1*

Purification steps	Protein[a] (mg)	Cytochrome *c*[b] (mg)	Concentration of cytochrome *c* (mg/mg protein)	Purification fold	Yield (%)
Crude extract	4795.0	27.3	0.0057	1.0	100.0
Soluble fraction	4111.0	26.5	0.0065	1.1	97.1
Ammonium sulfate (35-90%)	2435.0	16.0	0.0066	1.2	58.7
Cytochrome *c*-I					
DEAE-sephacel	498.0	1.0	0.0020	0.4	3.7
CM-cellulose	151.0	1.1	0.0073	1.3	4.0
Sephadex G-150	12.5	0.2	0.0160	2.8	0.7
Sephadex G-75	0.3	0.1	0.3333	58.5	0.4
Cytochrome *c*-II					
DEAE-sephacel	684.0	13.1	0.0190	3.3	48.0
1st CM-cellulose	375.0	9.9	0.0264	4.6	36.3
Sephadex G-150	52.5	6.4	0.1219	21.4	23.4
2nd CM-cellulose	23.4	3.4	0.1453	25.5	12.5
Sephadex G-75	9.8	3.2	0.3265	57.3	11.7
Cytochrome *c*-III					
DEAE-sephacel	684.0	13.1	0.0190	3.3	48.0
1st CM-cellulose	375.0	9.9	0.0264	4.6	36.3
Sephadex G-150	28.5	2.6	0.0912	16.0	9.5
2nd CM-cellulose	12.0	1.9	0.1583	27.8	7.0
Sephadex G-75	2.3	1.6	0.6957	122.1	5.9

[a] Estimated by the method of Lowry *et al.* (1951).
[b] Determined by the method of Tani *et al.* (1985).

with N_2 gas for 3 min was first reduced with a few crystals of sodium dithionite. One sample was then flushed with CO and the other which was used as a control was bubbled with N_2 gas for 30 min.

Electrophoresis

Polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS) in 12.5% acrylamide was performed by the procedure of Laemmli (1970). Proteins were stained with Coomassie Brillant Blue R-250 (Weber and Osborn, 1969).

Protein determination

Protein was determined by the method of Lowry *et al.*(1951). Bovine serum albumin was used as a standard.

RESULTS

Purification of soluble cytochrome *c*

As indicated in Table 1, three soluble *c*-type cytochromes (*c*-I, *c*-II, and *c*-III) which behaved homogeneously in SDS-PAGE were obtained. Because it was impossible to distinguish the three cytochromes in mixture, the accurate proportions of the three in the crude extract, soluble fraction, and ammonium sulfate fractionate could not be determined. When the three cytochromes were left under air, *c*-I was oxidized most rapidly.

Molecular weight

The molecular weights of the cytochrome *c*-I, *c*-II, and *c*-III were estimated to be 12,500, 16,000, and 16,000, respectively, by FPLC. SDS-PAGE gave comparable values of 13,000, 14,000, and 14,000 for *c*-I, *c*-II, and *c*-III, respectively, indicating that the proteins are monomers (Fig. 1).

Isoelectric point (pI)

Each of the purified cytochromes gave one band after analytical isoelectric focusing in polyacrylamide gel. The pIs of the cytochrome *c*-I, *c*-II, and *c*-III were 8.8, 6.6, and 6.6, respectively.

Absorption Spectra

Cytochrome *c*-I showed an absorption peak at 411.8 nm in the oxidized state, and three peaks at 416.6, 522.2, and 551.4 nm in the reduced state (Fig. 2a). The absorption spectrum of the pyridine ferrohemochrome of *c*-I showed three peaks at 417.4, 521.8, and 551.4 nm.

As shown in Fig. 2b, oxidized cytochrome *c*-II showed an absorption maximum at 409.0 nm. The

Fig. 1. *Mobilities of cytochrome c-I, c-II, and c-III on SDS-polyacrylamide gel.* The purified cytochrome *c*-I (0.7 μg), *c*-II (2.0 μg), and *c*-III (1.0 μg) were electrophoresed on 12.5% polyacrylamide gel. The standard marker proteins were bovine serum albumin (66,000), ovalbumin (43,000), carbonic anhydrase (29,000), and horse cytochrome *c* (12,400). Lane: a, cytochrome *c*-III; b, cytochrome *c*-II; c, cytochrome *c*-I; d, marker proteins.

reduced *c*-II, on the other hand, showed three peaks at 416.5, 521.6, and 551.0 nm. The pyridine ferrohemochrome of the cytochrome showed absorption peaks at 415.6, 521.2, and 550.2 nm.

Fig. 2c shows that cytochrome *c*-III shows an absorption peak at 410.2 nm when it is oxidized and three peaks at 416.0, 521.8, and 551.2 nm when it is reduced. The absorption spectrum of the pyridine ferrohemochrome of this cytochrome revealed peaks at 414.2, 521.6, and 550.0 nm.

Interaction with CO

The shift in the Soret maximum of cytochrome *c*-III after treatment with CO indicates that the cytochrome has high affinity to CO (Fig. 3c). The relatively slight shift in the Soret peak of the cytochrome *c*-I, on the other hand, indicates that the cytochrome has relatively lower affinity than *c*-III to CO (Fig. 3a). However, there was no detectable shift in the Soret peak of cytochrome *c*-II after exposure to CO (Fig. 3b), indicating that the cytochrome *c*-II may not bind CO.

The (reduced plus CO) minus reduced difference spectrum of the cytochrome *c*-III revealed three troughs at 417.5, 520, and 550.5nm (Fig. 4b). The spectrum of the cytochrome *c*-II, however, showed only a trough at 419.5 nm (Fig. 4a).

Several properties of soluble cytochrome *c* purified from cells of *Methylobacillus* sp. strain SK 1 grown on methanol are summarized in Table 2.

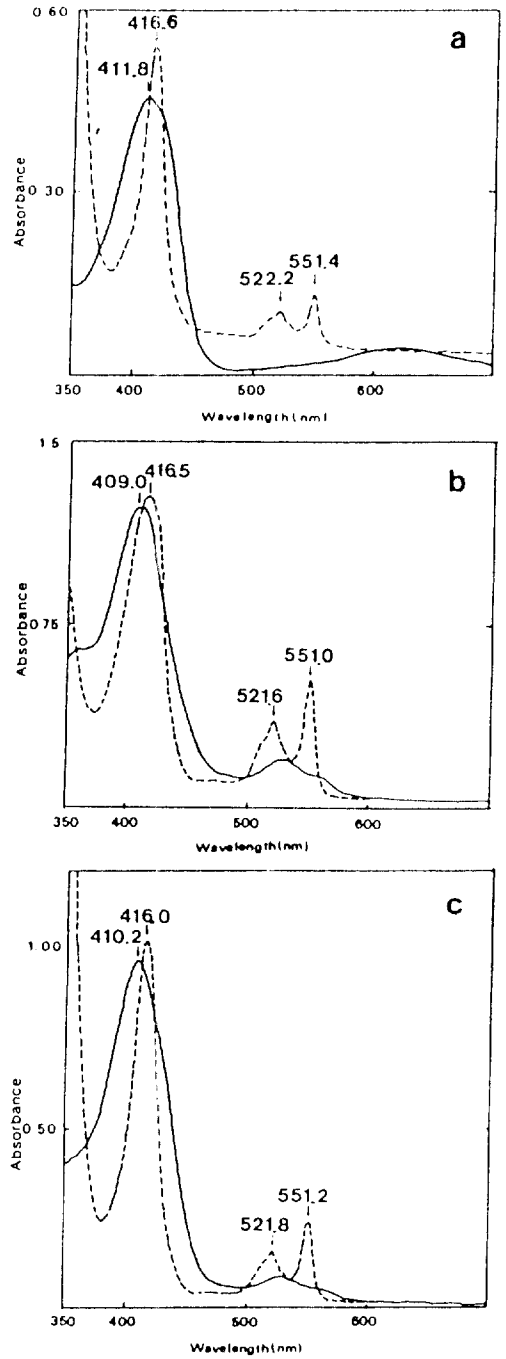

Fig. 2. *Absorption spectra of cytochrome c-I, c-II, and c-III.* Absorption spectra of purified cytochrome *c*-I (a) (0.034 mg/ml), *c*-II (b) (0.2 mg/ml), and *c*-III (c) (0.094 mg/ml) were obtained at room temperature as described in methods. Symbols:--------, reduced form; – – ——, oxidized form.

DISCUSSION

In recent years, methylotrophic bacteria have received a great deal of attention due to their commercial and biotechnological potentials such as single cell proteins, biopolymers, coenzymes, and cytochrome *c* (Anthony, 1982 and 1986; Tani, 1985). The successful use of these bacteria require a detailed knowledge of their physiology, biochemistry, and genetics. One aspect of this requirement is an understanding of the mechanism of methanol oxidation in these bacteria. Anthony (1986) suggested an electron flow in the respiratory chain during oxidation of methanol by methylotrophic bacteria, in the order of MeDH-cytochrome c_L-cytochrome c_H-terminal oxidase. However, the numbers and properties of *c*-type cytochromes were found to be different from each other, and some novel *c*-type cytochromes were also found in several methylotrophic bacteria.

It was reported that *Methylomonas* J contains two (*c*-551(I) and *c*-551(II))(Ohta and Tobari, 1981), *Methylomonas* sp. YK 56 has three (*c*-I, *c*-II, and *c*-III) (Tani *et al.*, 1985), and *Hyphomicrobium*

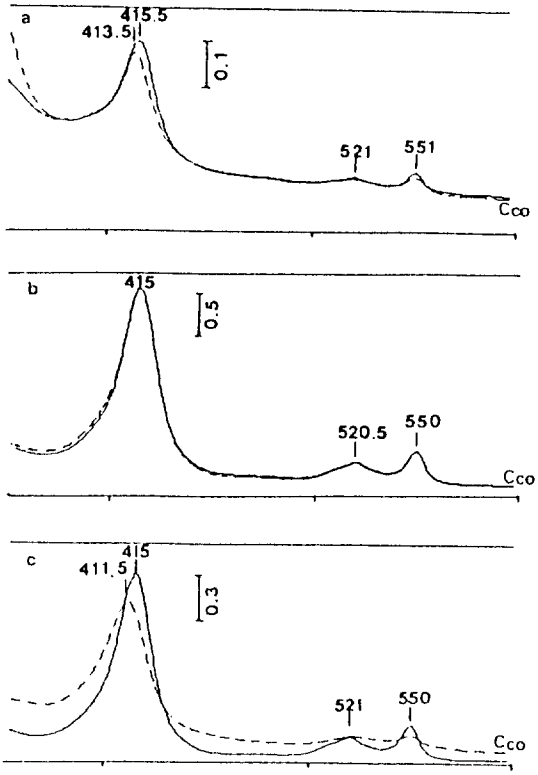

Fig. 3. *Reactions of cytochrome c-I, c-II, and c-III with CO.* a, cytochrome *c*-I (2.8 μM); b, cytochrome *c*-II (12.3 μM); c, cytochrome *c*-III (5.9 μM). Symbols: ———, reduced cytochromes ;------ -- C_{CO}. Cytochromes flushed with CO for 30 min after reduction with sodium dithionite.

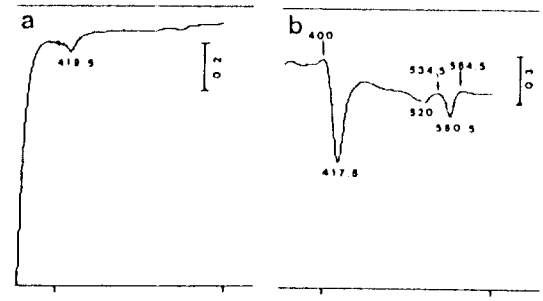

Fig. 4. *(Reduced plus CO) minus reduced difference spectra of cytochrome c-II and c-III.* a, cytochrome *c*-II (12.3 μM); b, cytochrome *c*-III (5.9 μM). Cytochromes reduced with sodium dithionite were bubbled with CO for 30 min.

Table 2. *Properties of soluble cytochrome c from Methylobacillus sp. strain SK1*

	c-I	*c*-II	*c*-III
Isoelectric point	8.8	6.6	6.6
Molecular weight			
Native	12,500	16,000	16,000
Denatured	13,000	14,000	14,000
Absorption maxima			
Ferrocytochrome (α, γ)	551.4, 416.6	551.0, 416.5	551.2, 416.0
Ratio of γ-/α-absorption (ferrocytochrome)	4.19	3.21	4.17
Absorption coefficient (mM $^{-1}$cm $^{-1}$) (α, γ)	47, 197	43, 137	41, 172
CO-binding	+	−	+
Autooxidizability	Fast	Very slow	Medium

Table 3. *Comparison of the soluble cytochrome c of methylotrophic bacteria*

	M. extorquens AM1[a]		*M. methylotrophus*[b]		*Methylomonas* J[c]		*Methylomonas* sp. YK 56[d]		*Hyphomicrobium* X[c]		*Methylobacillus* sp. SK1[f]		
	c_H	c_L	c_H	c_L	c-551(I)	c-551(II)	c-II	c-III	c_H	c_L	c-I	c-II	c-III
Proportion (crude extract)	72%	28%	50%	42%	73%	27%	71.5%	26.0%	73.5%	26.5%	n.d.[g]	n.d.	n.d
Molecular weight	11,000	20,900	8,500	17,000 -21,000	16,000	12,500	16,000	20,000	15,500	19,500	12,500	16,000	16,000
Isoelectric point	8.8	4.2	8.85	4.0-4.35	5.3	4.3	4.1	3.5	7.3-7.5	4.3	8.8	6.6	6.6
Absorption maxima													
Ferrocytochrome (α)	550.5	549	551.25	549.75	551	551	551.5	551.5	550.6	550	551.4	551.0	551.2
Ferrocytochrome (γ)	416.5	416	416.25	416	417	416	416.5	416	414	414	416.6	416.5	416.0
Ferricytochrome (γ)	410	410	408	410	409	409	410	410.5	408	408	411.8	409.0	410.2
Ratio of γ-/α-absorption (ferrocytochrome)	5.23	6.25	2.72	5.16			7.34	5.58			4.19	3.21	4.17

[a] Day *et al.* (1990) and O'Keeffe and Anthony (1980). [b] Cross and Anthony (1980) and Santos and Turner (1988). [c] Ohta and Tobari (1981). [d] Tani *et al.* (1985). [e] Dijkstra *et al.* (1988). [f] This study. [g] n.d.: not determined.

X contains two (c_H and c_L) *c*-type soluble cytochromes (Dijkstra *et al.*, 1988). Recently, *M. extorquens* AM1 was found to contain three soluble *c*-type cytochromes, one of which is a novel cytochrome *c*, cytochrome *c*-553 (Day *et al.*, 1990), and others are two classic *c*-type cytochromes (c_H and c_L) reported previously (O'Keeffe and Anthony, 1980). *M. methylotrophus* also has three types of cytochrome *c*: c_H and c_L, and a new cytochrome *c*″ (Cross and Anthony, 1980; Santos and Turner, 1988). It has generally been agreed that there are one constitutive cytochrome c_{550} and two soluble *c*-type cytochromes (C_{551i} and C_{553i}) induced during growth on methanol in *P. denitrificans* (Husian and Davidson, 1986). Cytochrome c_{553i} was assumed to be an electron acceptor for MeDH (Ferguson, 1988), and cytochrome c_{551i} to be an electron acceptor for the blue copper protein (amicyanin). Long and Anthony (1991), however, demonstrated that cytochrome c_{553i} is not the MeDH electron acceptor and that the only electron acceptor for MeDH is the cytochrome c_{551i}.

Three *c*-type soluble cytochromes were purified in this study from cells of *Methylobacillus* sp. strain SK1 which grows only on methanol. Some properties of cytochromes *c* from *Methylobacillus* sp. SK1 were found to be similar to those of other methanol-oxidizing bacteria (Table 3). As shown in Table 3, the molecular weights of *c*-type cytochromes with high pI in most methanol-oxidizing bacteria including *Methylobacillus* sp. strain SK1 are smaller than those that have low pI except in *Methylomonas* J. The absorption maxima of the γ-and α-band of the *c*-type cytochromes from *Methylobacillus* sp. strain SK1 also are similar to those of other methylotrophic bacteria. However, the pIs of low pI cytochromes (*c*-II and *c*-III) of *Mehylobacillus* sp. strain SK1 are much higher than those of low pI *c*-type cytochromes from other bacteria. This together with the fact that the molecular weights of the three purified cytochromes are different from the *c*-type cytochromes in other methanol-oxidizing bacteria indicates that each methylotrophic bacterium uses unique soluble *c*-type cytochromes during growth on methanol.

Although the molecular weight, pI, and absorption spectra of the oxidized and reduced form of *c*-II and *c*-III purified from *Methylobacillus* sp. strain SK1 are identical or very similar, the two could not be counted as an identical molecule since they were different in many properties such as the rate of oxidation, the ability to bind CO, and the absorption spectra after treatment with CO. We, however, can not exclude the possibility that the *c*-II and *c*-III are identical cytochromes since no *c*-III was found in *Methylobacillus* sp. strain SK1 grown on methanol in the absence of copper ion (data not shown). This together with the fact that the oxidized *c*-III was slightly blue, it may be possible to assume that the *c*-III is a

c-II which has bound copper ions. The copper ions bound to the *c*-II may caused the protein to be oxidized rapidly under air and to bind CO.

적 요

메탄올만 이용하여 성장하는 *Methylobacillus* sp. strain SK1에서 세 종류의 가용성 cytochrome *c*를 정제하였다. Cytochrome *c*-I은 일곱 단계를 거치면서 58.5배 정제하였고, *c*-II와 *c*-III는 여덟 단계를 통하여 각각 57.3배와 122.1배 정제하였다. 이들의 분자량은 *c*-II가 12,500 이었고, *c*-II와 *c*-III는 모두 16,000으로 나타났다. 환원형 스펙트럼상에서는 cytochrome *c*-I 이 551.4, 522.2 및416.6 nm, *c*-II는 551.0, 521.6 및 416.5 nm, 그리고 *c*-III 가 551.2, 521.8 및 416.0 nm 에서 cytochrome *c* 고유의 α, β, γ peak를 나타내었다. 반면, 산화형 스펙트럼에서는 cytochrome *c*-I, *c*-II, 그리고 *c*-III 가 각각 411.8, 409.0 및 410.2 nm 에서 peak를 보였다. 환원형 스펙트럼에서 α와 γ peak의 absorption coefficient는 *c*-I 이 47과 197 $mM^{-1}cm^{-1}$ 이었고 *c*-II 는 43 과 137 $mM^{-1}cm^{-1}$, *c*-III 가 41 과 172 $mM^{-1}cm^{-1}$ 이었다. 정제한 세 종류의 cytochrome *c* 의 isoelectric point 는 *c*-I이 8.8, *c*-II와 *c*-III는 모두 6.6으로 밝혀졌다. 이들 가운데 cytochrome *c*-I과 *c*-III는 일산화탄소와 결합하였으나 *c*-II의 경우에는 반응하지 않았다.

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