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Stable Isotope Labeled Cytochrome c_3 from *Desulfovibrio vulgaris* on a Defined Medium as Sole Nitrogen Source

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To obtain Cytochrome c_3 labeled with a stable isotope, the conditions of cultivation and the composition of medium for *Dv*MF were examined. The growth of *Dv*MF was steady and reproducible under purging with N_2 and under pH control. *Dv*MF was able to go on a defined medium without natural products. The composition of the medium containing a small amount of NH_4Cl as sole nitrogen source was established. Then, uniformly ^{15}N-labeled Cytochrome c_3 was obtained during the culture of *Dv*MF in a defined medium with $^{15}NH_4Cl$; it was confirmed by ^1H-^{15}N HMQC.

Keywords: Cytochrome c_3, ^1H-^{15}N HMQC, Stable isotope

Introduction

Cytochrome c_3 that is found in the sulfate-reducing bacteria of the genus *Desulfovibrio* (Postgate, 1984), is a small (14 kDa) tetraheme protein and functions as an electron carrier for hydrogenase (Tamura *et al.*, 1988). This protein is of great interest (Yagi *et al.*, 1983). Especially, the relationship between its structure and its redox behavior has been the subject of several physicochemical and spectroscopic studies (Higuchi *et al.*, 1984; Niki *et al.*, 1984; Fan *et al.*, 1990; Turner *et al.*, 1992). The determination of the solution structure of small protein has been done by isotope-edited NMR experiments (Marion *et al.*, 1989; Gooley *et al.*, 1990). However, there are no reports of stable isotope uniformly labeled cytochrome c_3. Therefore, it is necessary to establish methods for obtaining enough uniformly ^{15}N-labeled cytochrome c_3. *Desulfovibrio* sp. were grown on the published media containing a natural product, e.g., yeast extract and polypeptone (Kobayashi & Skyring, 1982; Postgate, 1984). Natural products consist of various amino acids, consequently, those media have not been suitable for stable isotope uniform labeling. In this work, we examined the conditions of cultivation and the composition of the medium with the intention of obtaining uniformly ^{15}N-labeled cytochrome c_3.

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Materials and Methods

Bacteria Strain *Desulfovibrio vulgaris* Miyazaki F (*Dv*MF) is the strain isolated by Iwasaki (Kobayashi & Skyring, 1982) and obtained from Prof. T. Yagi of Shizuoka University.

Culture media The media had the following composition (per liter distilled water): 0.5 g of KH_2PO_4; 1 g of NH_4Cl; 10 g of 70% (W/W) sodium lactate; 4.5 g of Na_2SO_4; 0.04 g of $CaCl_2 \cdot H_2O$; 0.06 g of $MgSO_4 \cdot 7H_2O$; 0.004 g of $FeSO_4 \cdot 7H_2O$; and 1 ml of resazurin solution (1 g/l). NH_4Cl was changed in the range of 0.1 to 1.3 g as required. A filtersterilized 10% (W/V) yeast extract (Difco) solution or a vitamin solution was added (10 ml/l) where indicated. The vitamin solution contained (per liter distilled water) : 2 mg of biotin; 1 mg of pyridoxine HCl; 5 mg of thiamine HCl; 5 mg of cyanocobalamine; 5 mg of *p*-aminobenzoic acid; 5 mg of thiotic acid. The pH was adjusted to 7.4 with 5 M NaOH.

Cultivation of bacteria *Dv*MF was grown anaerobically in a 1 l and a 70 l fermenter containing 900 ml and 50 l of medium, respectively. The growth of *Dv*MF in the test medium was examined after at least two serial transfers of the cells at the early stationary phase in the same medium. After inoculation (5% inoculum) the culture was continuously stirred at 100 rpm, purged with N_2 at 0.04 VVM (gas supply per media volume per min), and kept at 37°C.

Estimation of growth The estimation of growth by measuring the optical density was difficult since the culture was blackening by FeS precipitation. The growth was denoted by grams of protein from one liter of culture. The total growth was obtained by subtracted X_i from $X_f \cdot X_i$ was the concentration of cell protein at the time of inoculation. X_f was the maximum value of the concentration of cell protein. The concentration of cell protein was measured after these cultures were hydrolyzed in 0.2 M NaOH.

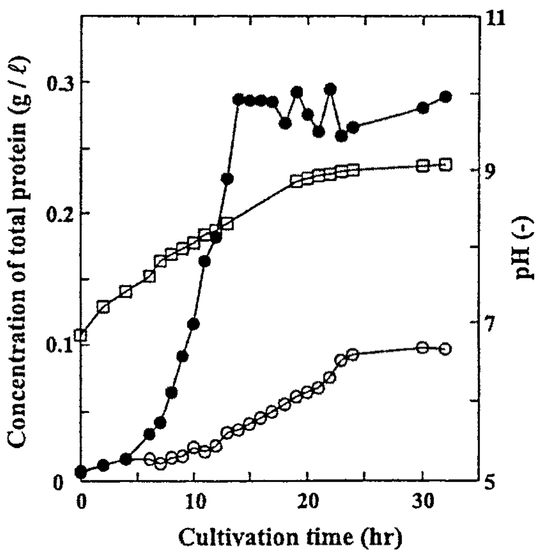

Fig. 1. Effects of purging with N_2 on growth of *Desulfovibrio vulgaris* Miyazaki F. The open and closed circles represent non-purging and purging with N_2, respectively. The open squares represent the pH of culture under purging with N_2.

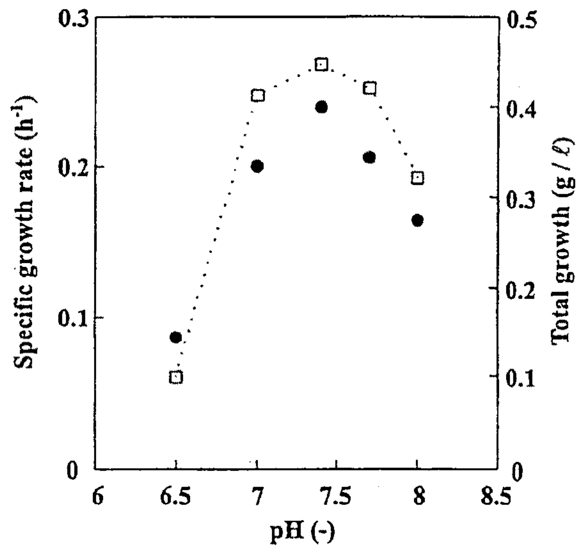

Fig. 2. Influence of pH on a specific growth rate and total growth. The closed circles represent the specific growth rate. The open squares represent the total growth. Full line show model fitting from the equation.

$\mu = \mu_{max} /(1 + H^+/K_H + K_{OH}/H^+)$

Where K_H and K_{OH} are rate constants.

NMR measurement Cytochrome c_3 was purified from *Dv*MF by the procedure described previously (Park *et al.*, 1991). The purity was confirmed by SDS-polyacrylamide gel electrophoresis. NMR spectra were obtained at pH 5.0 (30 mM phosphate buffer) and 30°C with a Bruker AM400 NMR spectrometer. The pulse sequence for heteronuclear multiple-quantum spectroscopy (HMQC) was referenced to Bax *et al.* (1983). Water suppression was achieved by presaturation during the relaxation delay.

Results and Discussion

Conditions of cullivation Fig. 1 shows the effects of purging with N_2 on the *Dv*MF growth. The specific growth rate is about to double and the total growth is 3 times, when the culture has been purged with N_2. The results are steady and reproducible. Reis *et al.* (1992) has reported that H_2S has a direct and reversible toxic effect on the other sulfate reducing bacteria. The above phenomenon is due to the elimination of H_2S by purging with N_2.

On the other hand, the pH of the Culture is led to alkalinity by eliminating H_2S. The influence of pH on the growth was examined by using an automatic pH-stat (1 M HCl) under N_2 purging. The dependence of a specific growth rate and the total growth on the pH of the culture are given in Fig. 2. As it can be observed, the total growth increases with the pH, reaching its maximum value, 0.448 g/l, at pH 7.4. The elevation of the specific growth rate with pH has the same pattern as the total growth. In fact, the maximum value of the specific growth rate is obtained at pH 7.4, $\mu = 0.24$ h^{-1}, equal to a mean doubling time of 2.9 h.

As can be observed in Fig. 2, a good fit (full line) of experimental results for the specific growth rate is obtained by using the inhibition model reported by Tang *et al.* (1989). The results suggest that hydrogen ion, H^+, is a growth inhibitor for the culture of *Dv*MF and the pH control is important for the *Dv*MF growth. Therefore, high cell densities are only reached by pH control and by the elimination of H_2S by purging with N_2.

Composition of mecthim *Dv*MF was grown in the presence of yeast extract (Park *et al.*, 1991). Yeast extract consists of various amino acids and vitamins. Consequently, the medium containing yeast extract has not been suitable for uniform labeling. It is necessary to determine the compositions of a medium as sole and small nitrogen sources for the tile purpose of the efficiency of ^{15}N uniform labeling. The effects of adding yeast extract to a growth medium is examined as further details oil the requirement of yeast extract is not examined for *Dv*MF (Table 1). In the presence of yeast extract, the specific growth rate and total growth are about 1.4 times that of the culture without yeast extract. While the specific growth rate and the total growth of the culture with vitamins is almost the same to that of the culture without adducts, it is not clear why yeast extract improves the growth; however, two interpretations can be offered: (1) To facilitate the synthesis of amino acids by the cells, yeast extract

Table 1. Effects of yeast extract and vitamins on growth.

Addition	Growth rate (h^{-1})	Total growth(g/l)
Yeast extract	0.24	0.448
Vitamins	0.19	0.316
None	0.17	0.314

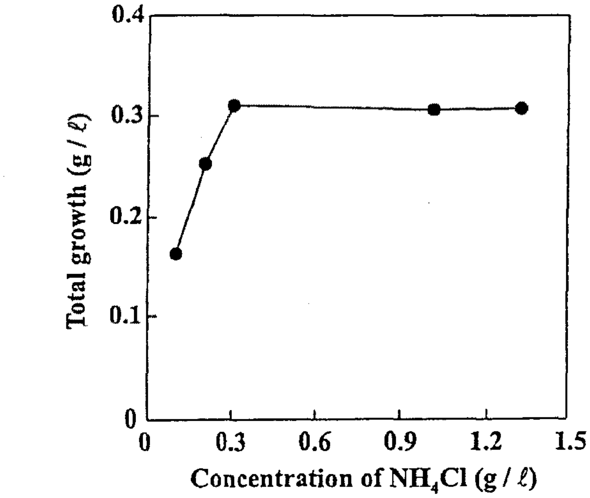

Fig. 3. Effects of ammonium chloride concentration on total growth in defined medium.

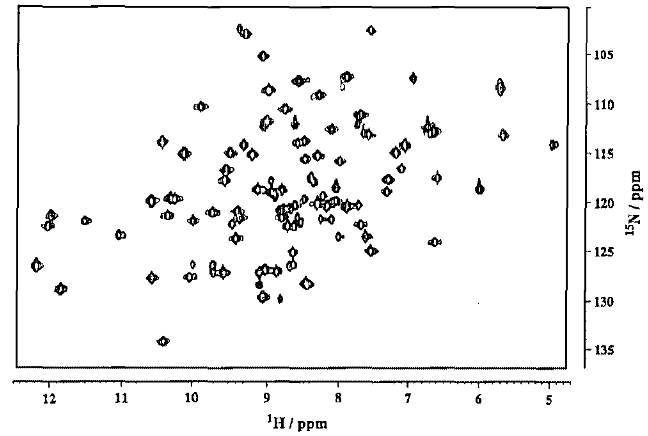

Fig. 4. Section of a ^{1}H-^{15}N HMQC spectrum of ferricytochrome c_3. The spectrum was acquired at 30°C, pH 5.0, in H_2O. The spectrum was acquired with spectral widths of 4000 Hz for ^{1}H and 5000 Hz for ^{15}N, 4 K data points in t_2, and 32 transients for each of $512t_1$ points. ^{1}H chemical shifts were referenced to the $^{1}H_2O$ signal at 4.73 ppm. ^{15}N chemical shifts were referenced to external saturated NH_4Cl at 27.34 ppm.

provides an exogenous supply of these acids: hence this enormously improved specific growth rate over the growth rate in the medium without yeast extract. (2) The presence of substances in the various amino acids, other acids which can be used as an energy source in addition to the lactate. The use of amino acids as energy substrates by *Desulfovibrio* strain has been especially reported (Stans *el al.*, 1985). As *Dv*MF does not essentially require yeast extract, we would hereafter grow *Dv*MF on the medium without yeast extract.

The cultures were grown under pH control (pH 7.4) in an anaerobic ferment on 62,5 mM lactate and 31.7 mM sulfate medium (containing 0.8 mg Fe^{2+} ion/l) with varying concentrations of ammonium chloride (Fig. 3). Ammonium chloride was found to be limiting for concentration up to 0.3 g/l. In these experiments, the ratio used on sulfate to lactate was 1:2. This value is keeping up with the overall equation for the oxidation of lactate by sulfate (Khosrovi & Miller, 1975).

$$2CH_3CHOHCOO^{-}+SO_4^{2-}\rightarrow 2CH_3COO^{-}+2CO_2+2H_2O$$

In addition, the growth of is reproducible. Therefore, 0.3 g/l is the concentration of ammonium chloride that was the minimum requirement of nitrogen substrate for the cells to grow under N_2 purging and under pH control.

We decided the composition of medium and the conditions of cultivation from these results. The defined medium has the following composition (per liter distilled water): 0.5 g of KH_2PO_4; 0.3 g of NH_4Cl; 10 g of 70% (W/W) sodium lactate; 4.5 g of Na_2SO_4; 0.04 g of $CaCl_2 \cdot 2H_2O$; 0.06 g of $MgSO_4 \cdot 7H_2O$; 0.004 g of $FeSO_4 \cdot 7H_2O$. The culture was continuously stirred at 100 rpm, purged with N_2 at 0. 04 VVM and kept at pH 7.4 and 37°C.

Production of uniformly ^{15}N-labeled cytochrome c$_3$ Uniformly ^{15}N-labeled cytochrome c_3 has never been obtained. The uniformly ^{15}N-labeled cytochrome c_3 would be obtained by replacing nitrogen in the above defined medium with $^{15}NH_4Cl$ (^{15}N; 99.7% Isotec). We obtained 1.82 μmol (25.3 mg) of cytochrome c_3 from 97.6 wet grams of *Dv*MF grown in 50 l of the defined medium. Tsuji and Yagi reported that the yields of cells and proteins were 17.7-26.0 wet grams and 55.5-70.9 nmol in 26 l of the peptone medium (Tsuji & Yagi, 1980). Therefore, Our results are satisfactory. Figure 4 shows the ^{1}H-^{15}N HMQC spectrum of uniformly ^{15}N-labeled cytochrome c_3. We observed at least 102 signals in Fig 4. *Dv*MF cytochrome c_3, composed of 107 amino acids containing 4 proline (Shinkai *et al.*, 1980), had 102 backbones NH. This result indicates that cytochrome c_3 is uniformly labeled with ^{15}N.

The conditions of cultivation and the composition of medium to obtain uniformly ^{15}N-labeled cytochrome c_3 from *Dv*MF are established in this work. This defined medium is a tool not only to obtain a stable isotope labeled protein but also to research biochemical properties of *Desulfovibrio* sp. Our next target is to make sequential assignment of cytochrome c_3 on the basis of uniformly ^{15}N-labeled cytochrome c_3 obtained in this work.

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