大腸菌におけるトリメチルアミンN-オキサイド還元酵素の誘導

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Induction of Trimethylamine N-oxide Reductase in *Escherichia coli*

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The addition of trimethylamine N-oxide (TMO) to a culture medium containing glucose, casamino acids, yeast extracts and inorganic salts promoted the growth of *Escherichia coli*. No further breakdown of trimethylamine which is formed from TMO was observed during the period of cell growth. The addition of the oxide resulted in a remarkable increase in TMO reductase levels in cells, depending on the amount added to the culture. The increase was attributable to induced synthesis of the reductase as evidenced by the prevention of *de novo* synthesis of the enzyme protein in the presence of actinomycin D and chloramphenicol.

Concerning accumulation of trimethylamine N-oxide (TMO) in relatively high concentration in the muscles of marine fish and invertebrates,[1] enormous amounts of reports have been presented. It is also well known that the oxide is reduced to trimethylamine (TMA) mainly by bacterial action during spoilage of fish.[2] However, there can be found few information about precise mechanisms of the reduction of TMO in bacterial cells.

As one of the characteristics of the enzyme responsible for the reduction, the increased activity was already reported when TMO was added as a substrate of the reaction into the culture media of some species of bacteria.[3-5]

The present study was undertaken to confirm the stimulation of the enzyme activity by TMO during cultivaion of *Escherichia coli*. Emphasis was placed on elucidating the increase in the activity resulted from *de novo* synthesis of the enzyme protein, generally called as induction of the enzyme in bacterial cells.

Material and Methods

Organism and growth condition *Escherichia coli* (IFO 3301) was grown statically at 37°C in a medium (1.0 *l*, pH 6.9) containing 2.0% glucose, 0.8% casamino acids, 0.25% yeast extracts, 0.1% NaCl, 0.28% $MgSO_4 \cdot 7H_2O$, and 0.4% K_2HPO_4. Unless otherwise mentioned, TMO was added to the culture at the concentration of 7.3×10^{-2} M when cells reached to 0.43–0.48 of optical density at 660 mμ. After an aliquot withdrawn from the culture was immediately cooled by being poured onto the frozen buffer of 0.04 M phosphate (pH 7.1), cells were collected by centrifugation. Cells were washed twice with

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the same buffer and suspended in 0.1 M phosphate buffer (pH 7.1). Cell density was measured at 660 mμ in a 1 cm cuvette on a spectrophotometer, Shimadzu D-40D. Dry cell weight was estimated after cells were washed with water and dried at 105°C for 3 hr.

Assay of TMO reductase activity Assay mixture consisted of 25 μmole methylviologen, 50 μmole TMO, 320 μmole phosphate buffer, and cell suspension (2.5–5.0 mg dry cells) in 3 ml of a total volume (pH 7.1). The reaction was started by adding 8.6 μmole of $Na_2S_2O_4$ to assay mixture. Incubation was carried out at 37°C and the enzymic reaction was stopped by adding 2 ml of 10% trichloroacetic acid. An aliquot of this mixture was submitted to determination of TMA[6] which was produced by the bacterial enzyme action.

Determination of residual TMO and total TMA in the culture Residual TMO and total TMA in the culture were determined by the method modified by TOZAWA *et al.*,[7] after reducing TMO to TMA by the method of BYSTEDT *et al.*[8]

Results and Discussion

Effect of TMO on the growth of cells In order to examine the effect of TMO on the growth of cells, the oxide was added to the culture at 0 and 55 min. As the result given in Fig. 1, TMO was found to promote the bacterial growth in both of the cases. The lengths of the period which was required for appearance of the promotion were about 25 and 15 min, respectively, becoming shorter when TMO was fed to aged cells than to young cells.

The promotion of the growth is supposed to have resulted from utilizing TMO as a carbon and/or a nitrogen source for the bacteria. The possibility, however, could be excluded from the result that TMA formed was not decomposed further, as indicated in Fig. 2. The amount of total TMA determined after residual TMO in the culture was

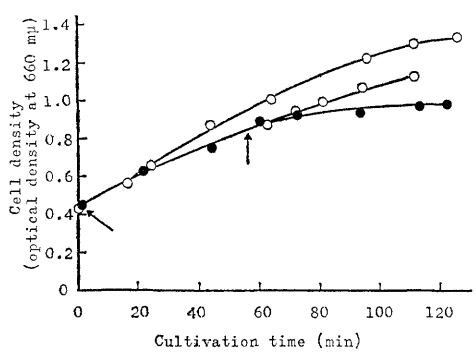

Fig. 1

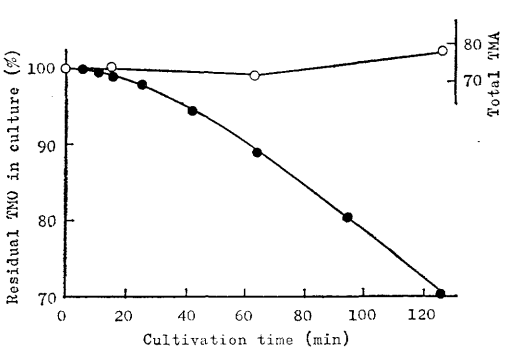

Fig. 2

Fig. 1. Effect of TMO on growth of cells. Trimethylamine N-oxide was given to the culture at 0 and 55 min, as shown by arrows.
○—○, with TMO; ●—●, without TMO

Fig. 2. Change in amounts of residual TMO and total TMA in the culture.
●—●, residual TMO; ○—○, total TMA (μmole/ml culture)

entirely reduced by means of titanium trichloride, did not show any change at all during the cultivation, although 30% of TMO initially added was reduced to TMA in the cultivation period of 120 min. The evdence thus observed suggests that TMO does not promote the bacterial growth either as a carbon or a nitrogen source under the present culture condition. Oxygen in TMO molecule seems to take part in the stimulation of the growth, possibly as a terminal electron acceptor. This situation can be understood to be closely analogous to that in the reduction of nitrogenous oxides in bacterial cells.[9,10]

Induction of TMO reductase by TMO The effect of TMO on TMO reductase activity of cells was examined by adding the oxide to the culture at 0 and 55 min. The result can be seen in Fig. 3, indicating that cells in the culture lacking TMO exhibited no increased activity at all. In contrast, cells fed TMO at 0 min as well as at 55 min revealed the dramatic increase in the activity after short lag periods.

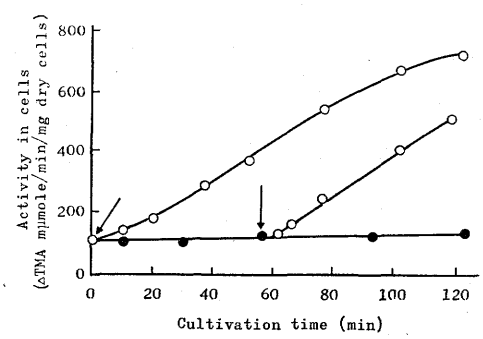

Fig. 3

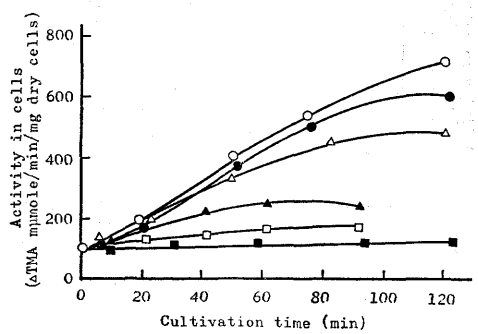

Fig. 4

Fig. 3. Effect of TMO on the enzyme level in cells.
Trimethylamine N-oxide was given to the culture at 0 and 55 min, as indicated by arrows.
○—○, with TMO; ●—●, without TMO

Fig. 4. Effect of TMO with varied concentrations on the enzyme levels of cells.
●—●, 2.2×10^{-1} M; ○—○, 7.3×10^{-2} M; △—△, 7.3×10^{-3} M; ▲—▲, 2.2×10^{-3} M; □—□, 7.3×10^{-4} M; ■—■, 0 M

The increase in the activity was tested at various concentrations of TMO in the culture. The result is shown in Fig. 4. The activity increased according as TMO was supplemented highly to the culture: 7.3×10^{-2} M showed the maximum activity, while 2.2×10^{-1} M did rather inhibitory effect.

The greater part of previous investigations[3-5] on bacterial TMO reductase have simply dealt with the increase in the enzyme activity by adding TMO to culture media, and they did not present any evidence for stimulation of the reductase formation. We made an attempt to elucidate this point by blocking *de novo* synthesis of the enzyme, using two kinds of antibiotics, actinomycin D and chloramphenicol.

Actinomycin D (Sigma Chemical Co.), which is known as a potent inhibitor of DNA-dependent RNA synthesis preceding *de novo* synthesis of proteins,[11] was given together

with TMO to the culture. Cells were treated previously with ethylenediaminetetraacetate (EDTA) so that the antibiotic can permeate into cells.[12] The result, as shown in Fig. 5, indicates that actinomycin D highly prevented the enzyme activity which was revealed by the addition of TMO. The prevention may possibly have resulted from cessation of the *de novo* enzyme synthesis. As compared with the activity in Fig. 3, the more decreased activity of cells fed TMO was found in this experiment, probably be due to the treatment with EDTA. Chloramphenicol (Sankyo Co. Ltd.) given to the culture at 45 min also exhibited significant inhibition of the enzyme activity, as given in Fig. 6. The inhibition by the antibiotic which is recognized to relate the interference of bacterial ribosome function[13] appears to have resulted from inhibiting the *de novo* enzyme synthesis. Incidentally, the slightly decreased activity was observed after the addition of the antibiotic to the culture (Fig. 6). A concentration of the antibiotic adopted in the present experiment (144 μg/ml culture) may not be enough to prevent the bacterial growth while sufficient to interfere the enzyme formation. In order to make the situation clear, the enzyme activity was plotted against the cell density per unit volume

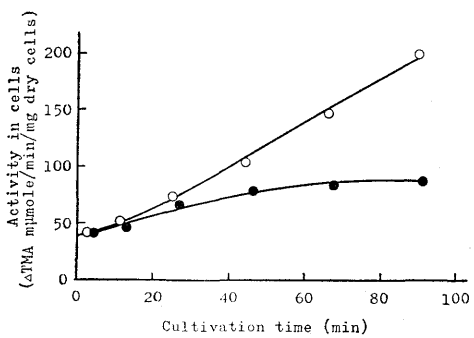

Fig. 5

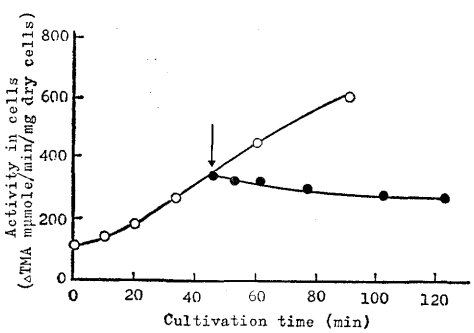

Fig. 6

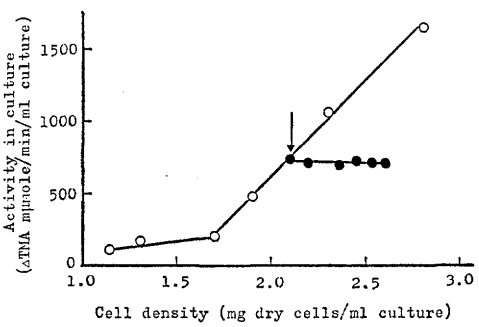

Fig. 7

Fig. 5. Inhibition of the enzyme formation in cells by actinomycin D.
After cell density reached 0.40 of optical density at 660 mμ, EDTA was added to the culture at 3.3×10^{-2} M, followed by incubation for 3 min. Actinomycin D (10 μg/ml culture) together with TMO was given to EDTA-treated cells in the culture. ●—●, with antibiotics; ○—○, without antibiotic

Fig. 6. Inhibition of the enzyme formation in cells by chloramphenicol,
Chloramphenicol (144 μg/ml culture) was given at 45 min after the addition of TMO, as indicated by arrow. ●—●, with antibiotic; ○—○, without antibiotic

Fig. 7. Differential plot of the enzyme activity versus cell density in the culture.
At the point indicated by arrow chloramphenicol was added to the culture. ●—●, with antibiotic; ○—○, without antibiotic

of the culture, as drawn in Fig. 7. The enzyme in cells grown before the addition of the antibiotic was kept at a constant level, even though the bacterial growth occured thereafter. This indicates that chloramphenicol affected the formation of the reductase protein more preferentially than protein synthesis with which the constitutive growth of cells is concerned. The observation seems to be in accord with the finding of some inducible or repressible enzymes in *E. coli.*[14]

It comes to conclusion that the remarkable increase in the activity by TMO is attributable to the *de novo* synthesis of the enzyme protein, in another word, *induction* of TMO reductase in *E. coli* cells.

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