Induction of Dibenzothiophene Oxidation by Pseudomonas jianii

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Dibenzothiophene (DBT) was oxidized with supplement of energy source such as lactate or glycerol by resting cells of Pseudomonas jianii. Necessity of the supplement was in agreement with the phenomenon of co-metabolism. DBT oxidizing enzymes were induced by benzene ring having no side chain such as DBT, naphthalene, and anthracene. Supplemental substance, co-substrate, was used as energy source for synthesis of the enzymes. Induction of the enzymes was significantly repressed by presence of glucose, and the repression could be overcome by addition of cyclic-3', 5'-adenosine monophosphate (cAMP).

DBT was oxidized to 3-hydroxy-2-formylbenzothiophene (HFB), and addition of meat extract was necessary for the oxidation of DBT by growing cells of Ps. jianii or Ps. abikonensis as an organic nitrogen source.1~4)

This report deals with the necessity of organic substances in DBT oxidation and induction of DBT oxidizing enzymes by using resting cells of Ps. jianii. Manometric method was used in this study with supplementing some carbon compounds as organic substances.

MATERIALS AND METHODS

Microorganisms and cultivation. Resting cells of Ps. jianii strain DDC 279 were used in this study. The cells were cultivated with meat extract medium or lactate medium at 30°C under the shaking condition (120 rpm) for 3 days. After the cultivation, cells were harvested by centrifugation at 10,000 x g for 5 min, and washed with 0.02 M phosphate buffer (pH 6.5). The meat extract medium had the following composition (per liter of distilled water): MgCl2·6H2O, 0.2 g; KH2PO4, 1.4 g; Na2HPO4·12H2O, 9.5 g; meat extract, 10 g; pH 7.3. The lactate medium had the following composition (per liter of distilled water): lactic acid, 20 g; NH4Cl, 5 g; KH2PO4, 1.4 g; Na2HPO4·12H2O, 9.5 g; MgCl2·6H2O, 0.2 g; CaCl2, 5 mg; ZnSO4·7H2O, 12.5 mg; FeSO4·7H2O, 12.5 mg; MnSO4·3H2O, 12.5 mg; pH 7.3.

Measurement of oxygen uptake. The amount of oxygen uptake by the resting cells was measured manometrically by the Warburg technique5) at 30°C under the shaking condition (120 rpm). Cell suspension and substrate were added in ca. 20 ml volume of Warburg flask, and final volume was brought to 2.0 ml with the phosphate buffer. Center well contained 0.2 ml of 15% KOH solution.

Preparation of cell suspension and adaptation to DBT. Washed cells were suspended in the phosphate buffer, and the cell suspension was used as DBT unadapted resting cells. A resting cell suspension adapted to DBT was obtained by incubation of the cells with ca. 1 mM of DBT and 0.7~1.4 mM of lactate or glycerol at 30°C for 40 min, and washed with the phosphate buffer, and suspended in the phosphate buffer.

Determination of oxidized products from DBT. Qualitative analyses of DBT oxidation were carried out by vermilion coloring of one of the products, trans-4[(2-(3-hydroxy)-thionaphthenyl)-2-oxo-3-butenolic acid,4) and by FeCl3 test6) for HFB. Quantitative analysis of DBT oxidation was carried out by measuring optical density at 390 nm which showed amount of HFB. HFB was the main product formed from DBT.3)

Reagents. DBT was obtained from Aldrich Chemical: benzothiophene, Fujisawa Pharmaceutical; cAMP and puromycin, Sigma Chemical; chloramphenicol, Sankyo.

RESULTS

Oxidation of supplemental substances and DBT

Oxygen uptake due to the oxidation of DBT was not observed by DBT unadapted
FIG. 1. Effect of Lactate and Chloramphenicol on the Oxidation of DBT by Cells Grown in Lactate Medium.
Cells, 18.0 mg (dry weight). Lactate, 2.8 μmole. Chloramphenicol, 0.6 μmole. Symbols: □—□, DBT; ○——○, lactate; •—•, DBT, lactate and chloramphenicol. Endogenous value was subtracted.

FIG. 2. Effect of Glucose on the Oxidation of DBT by Cells Grown in Lactate Medium.
Cells, 19.5 mg (dry weight). Symbols: •—•, 1.4 μmole of glucose; ○——○, glucose and 2.6 μmole of DBT. Endogenous value was subtracted.

resting cells in the absence of organic supplemental substance, but larger amount of oxygen consumption than the oxidation of lactate was observed 5 to 10 min after the start of incubation supplemented with lactate as shown in Fig. 1. After the incubation, observation of coloring and FeCl₃ test were carried out in the mixture used. From the results, accumulation of HFB was not detected in the mixture which showed no oxygen uptake other than endogenous respiration, but the accumulation of HFB was observed in the mixture which showed excess oxygen uptake on the oxidation of lactate by the coloring and the positive reaction to FeCl₃ test. Twenty-three hr after the start of the incubation supplemented with lactate, the excess oxygen uptake on the oxidation of lactate showed 4.4 μmoles of oxygen amount per μmole of accumulated HFB. On the other hand, four point eight (19/4) of molar ratio of oxygen consumption to HFB was obtained by the calculation of the following equation:

\[
4C₆H₅S + 19O₂ = 4C₆H₅O₃S + 12CO₂ + 6H₂O
\]  
(DBT)  
(HFB)

The approximate agreement in the molar ratio mentioned above also exhibited that the excess oxygen uptake was shown as a result of the oxidation of DBT. However, buffer solution and vessels prepared were sterilized for preventing from infection especially in this experiment.

Chloramphenicol treatment for resting cells grown in lactate medium inhibited the activity of DBT oxidation, but lactate oxidation was unaffected as shown in Fig. 1. The same phenomena mentioned above were observed also by the addition of puromycin.

In the oxidation of DBT by DBT unadapted resting cells, glycerol was also available and showed similar time course of the oxygen consumption as exemplified in Fig. 1. On the contrary, supplement of glucose was ineffective for the oxidation of DBT within about 60 min from the end of glucose consumption as shown in Fig. 2.

**Effect of preincubation on DBT oxidation**

Figure 3 shows effect of supplemental substances, lactate, glucose, and cAMP, in the preincubation. The supplemental substances in the preincubation were eliminated by washing after the preincubation. First, no oxygen uptake was observed by cells preincubated with only DBT. The second, an immediate oxygen uptake was observed by cells preincubated with DBT and lactate, and
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Effect of Preincubation on the Oxidation of DBT.

Oxygen uptake due to the oxidation of DBT was measured. DBT, 2.6 µmole. Cells, 16.3 mg (dry weight). Endogenous values were subtracted. Cells used were cultivated with meat extract medium and washed after preincubation with the following substances at 30°C for 40 min. Preincubated with 0.9 mm of DBT (●—●); DBT and 1.4 mm of lactate (○—○); DBT, 0.7 mm of lactate and 0.35 mm of glucose (■—■); DBT, 0.35 mm of glucose and 2 mm of cAMP (□—□).

Effect of Chloramphenicol on the Adaptation to DBT Oxidation.

Warburg flask contained 18.0 mg (dry weight) of cells, 2.6 µmole of DBT and 0.6 µmole of chloramphenicol. Endogenous values were subtracted. Cells used were cultivated with meat extract medium and washed after preincubation with the following substances at 30°C for 40 min respectively. Preincubated with 1.4 mm of lactate and 1.3 mm of DBT (□—□); lactate, DBT and 0.3 mm of chloramphenicol (●—●); lactate (□—□).

Discussion

Although DBT unadapted resting cells of *P. jianii* were unable to oxidize DBT without supplemental substances, excess of oxygen uptake on the oxidation of supplemental substance was observed several minutes after the addition of effective organic energy source also an immediate oxygen uptake was observed by cells preincubated with DBT, glucose, and cAMP. The third, an oxygen consumption with evidently long lag time was shown by cells preincubated with medium containing glucose.

Figure 4 shows effect of chloramphenicol on adaptation to DBT oxidation of cells grown in lactate medium. Although no DBT oxidation was observed in the cells preincubated with only lactate, DBT oxidation occurred even in the presence of chloramphenicol when cells were preincubated with DBT and lactate. However, DBT oxidizing activity was lost when chloramphenicol was present during the preincubation with DBT and lactate. The same phenomena mentioned above were also shown by the addition of puromycin.

As is shown in Table I, some substances induced the oxidation of DBT. Supplemental substance used in preincubation was glycerol. The immediate oxidation of DBT, without lag, was shown by cells preincubated with benzene, naphthalene, anthracene, or HFB.

<table>
<thead>
<tr>
<th>Inducer</th>
<th>Oxidation of DBT</th>
<th>Inducer</th>
<th>Oxidation of DBT</th>
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</thead>
<tbody>
<tr>
<td>None</td>
<td>−</td>
<td>Toluene</td>
<td>−</td>
</tr>
<tr>
<td>DBT</td>
<td>+</td>
<td>o-Xylene</td>
<td>−</td>
</tr>
<tr>
<td>Benzene</td>
<td>+</td>
<td>Thiophene</td>
<td>−</td>
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<tr>
<td>Naphthalene</td>
<td>+</td>
<td>Benzothiophene</td>
<td>−</td>
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<tr>
<td>Anthracene</td>
<td>+</td>
<td>HFB</td>
<td>+</td>
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Table I. Compounds Effective as Inducer in the Oxidation of DBT
such as lactate (Fig. 1). The excess oxygen consumption showed the oxidation of DBT by the quantitative analysis of HFB in the mixture. Thus, DBT was unusable as a carbon source for DBT unadapted cells, and necessity of the supplemental organic energy source was showed in agreement with the phenomenon of co-metabolism. Co-metabolism was first reported by Leadbetter and Foster\(^7\) as a term of co-oxidation when they noted microbial oxidation of ethane in the presence of methane. The term of co-metabolism implying co-oxidation conveys oxidation of substances by the presence of usable carbon source \textit{i.e.}, co-substrate. Therefore, lactate or glycerol supplemented was used as a co-substrate for DBT oxidation.

Immediate oxidation of DBT by cells preincubation with DBT and lactate (Fig. 3) demonstrated that the DBT oxidizing enzymes were induced by DBT. The following fact suggests that the DBT oxidizing enzymes were induced with benzene ring having no side chain; \textit{i.e.}, DBT was oxidized without lag by cells adapted to benzene, naphthalene, or anthracene (Table I). Moreover, inability of thiophene ring as an inducer is suggested by the fact that the cells preincubated with thiophene or benzo thiophene failed in DBT oxidation without lag. The inability of thiophene ring for the induction is suggested also by DBT oxidation pathway which stops on the way and results in the accumulation of HFB.\(^4\) Induction of DBT oxidizing enzymes was also shown by the result that ability of unadapted cells on DBT was obstructed by the treatment with chloramphenicol (Figs. 1 and 4).

Some workers reported that glucose is a nonrepressing co-substrate, for example, in the degradation of dodecylbenzene sulfonate by enrichment culture\(^6\) and the degradation of chlorobenzoate by activated sludge.\(^9\) In this study, supplemental substances showed to have differences in the lag time, and glucose was found to be a repressor as a co-substrate for the oxidation of DBT, and DBT was not oxidized during presence of glucose in the incubation mixture. The fact that the long lag caused by glucose was overcome with the addition of cAMP (Fig. 3) showed the existence of catabolite repression between the synthesis of the DBT oxidizing enzymes and glucose. Catabolite repression was first reported by Magasanik,\(^10\) and the relation between glucose and cAMP was demonstrated by a number of workers.\(^11\) The necessity of co-substrate on DBT adaptation demonstrated that co-substrate was used as an energy source for the synthesis of the inducible enzymes in DBT oxidation by DBT unadapted resting cells. From the above results, it was confirmed that DBT was practically unusable as a carbon source for energy and was unable to induce specific enzymes.

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