Studies on Diaminododecane Utilization by Bacteria

(Part II) Studies on Diaminododecane Utilization by *Corynebacterium* sp. DAD 2-3

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Diaminododecane 자화균에 관한 연구

(제 2 보) *Corynebacterium* sp. DAD 2-3의 Diaminododecane자화에 관한 연구

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ABSTRACT

A *Corynebacterium* sp. capable of utilizing diaminododecane (DAD) were isolated from the soil by enrichment culture. Among 9 different kinds of substituted alkanes containing CN, NH_2, Cl, and SH groups (monoterminally or diterminally substituted) tested as carbon source, the isolate, designated as DAD 2-3, utilized DAD, putrescine dihydrochloride, dodecane and laurylamine. Dodecanethiol, thioanisole, decanedithiol, dicyanooctane, laurylcyanide,and dichlorodecane were not utilized. When emulgen 950 was added to the medium, the growth of DAD 2-3 was slightly accelerated. Isolate DAD 2-3 grown in the medium with DAD as carbon source formed α-ketoglutaric acid. Metabolic product of DAD 2-3 grown in a medium without nitrogen source was different from that of grown in a medium with NH_4NO_3. When glucose, putrescine, n-dodecane and other alkane derivatives were tested in place of DAD, isolate DAD 2-3 yielded products different from those they formed with DAD suggesting specificity of DAD as a carbon source.

INTRODUCTION

Long chain linear alkylamines and their salts have been widely used as finishing or dyeing agents in the textile industry and as a few materials of cationic or amphoteric surfactants. The increasing amounts of production and use of these alkylamine derivatives make it more important to assess their biodegradability from the ecological point of view(Yoshimura et al., 1980).

Many studies on the degradation of amines have been made (Colby et al., 1974; Desa, 1972; Durham et al., 1978; Eady et al., 1968; Large, 1971; Murooka et al., 1979; Yamada et al., 1965) but little is known about the degradation of long chain alkylamine (Yoshimura et al., 1980).

As reported in the previous paper(Lee, 1982), DAD-utilizing organisms were isolated from soil by enrichment culture. One of these, *Corynebacterium* strain DAD 2-2, was employed to study on the degradation of DAD.

This paper describes the degradation of DAD by the other strain which was isolated in a previous study.

MATERIALS AND METHODS

Isolation and Identification of Microorgaisms

The enrichment culture medium (DAD medi-

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um) contained the following ingredients (grams per liter) : DAD, 2.0; KH_2PO_4, 1.5; $Na_2HPO_4 \cdot 12H_2O$, 1.5; NH_4NO_3, 4.0; $MgSO_4 \cdot 7H_2O$, 0.001; $FeSO_4 \cdot 7H_2O$, 0.005; $CaCl_2 \cdot 2H_2O$, 0.01; yeast extract, 0.005. The pH of the DAD medium was adjusted to 7.0 before autoclaving. Bacteria capable of utilizing DAD as carbon source were isolated the same methods in a previous study (Lee, 1982).

Morphological and physiological characteristics of the isolated microorgainsms were examined following the guides of Bergey's Manual of Determinative Bacteriology (1974) and Komagata et al. (1969; 1970;1972). Meso-diaminopimelic acid was detected by TLC (Staneck, 1974).

Growth Test

Growth test was carried out in test tubes each containing 10ml medium without DAD but with 2% of the various alkane derivatives at 30°C under continuous reciprocal shaking. The effects of emulgen 950 was carried out in 500ml Sakaguchi flasks containing 100ml of DAD medium and 25 ppm emulgen. Growth was estimated by measuring the absorbance at 660nm using a Hitachi 124 spectrophotometer (Hitachi,Ltd. Tokyo, Japan).

Thin Layer Chromatography (TLC)

All preparative and analytical thin layer chromatography studies were conducted with commercially prepared silica gel 60 plate (Merch). The solvent systems used for preparative and analytical TLC were: (i) benzene-1,4 dioxane-acetic acid (90 : 25 : 4), (ii) n-butanol-acetic acid-distilled water (60 : 20 : 15). In the analytical TLC studies, the compounds were visualized by spraying the plates with 10% H_2SO_4 and charring at 150°C. The plates were also sprayed with ninhydrin, bromphenol blue, 2,4-dinitrophenylhydrazine,and triphenyltetrazolium chloride to determine the character of the unknown compounds.

Products from DAD

To obtain acidic products, the organism was grown to early stationary phase in a 500ml Sakaguchi flasks each containing 100ml of DAD medium at 30°C with reciprocal shaking. The supernatant obtained by centrifugation at 10,000 ×g for 20 min was acidified to pH 2.0 and extracted in a previous study (Lee, 1982). The acidic fractions obtained were used for TLC.

For the purification and identification of products, the isolate DAD 2-3 was grown to early stationary phase in 1 liter of the DAD medium at 30°C on the rotary shaker. Acidic fractions were obtained as described above. Purification of the acidic fraction was carried out by column chromatography. Column chromatography was used with silica gel 60 (column size: 1.8×25 cm, solvent system: cyclohexane-ethylacetate-acetic acid (60 : 20 : 4). The major component of the solvent fraction obtained by column chromatography was confirmed by TLC. The solvent fractions confirmed were collected and concentrated. The component thus purified was analyzed by TLC, gas chromatography, GC-mass spectrometer.

In examining the effect of eliminating the nitrogen source on the kind of degradation product, the isolate DAD 2-3 was grown in 100ml DAD medium without NH_4NO_3 at 30°C under reciprocal shaking. Acidic fractions were obtained as described above and used for TLC.

Products from Glucose, Putrescine and n-Dodecane.

To investigate the products formed from glucose, putrescine and n-dodecane, the isolate DAD 2-3 was grown to early stationary phase in 500ml Sakaguchi flasks each containing 100 ml medium without DAD but with 0.1% glucose, putrescine, and n-dodecane. Acidic fractions formed by isolate DAD 2-3 were obtained as described in a previous report (Lee, 1982). The acidic fractions obtained were used for TLC.

Products from Alkane Derivatives

Cells grown to early stationary phases in 100 ml nutrient broth at 30°C were harvested by cen-

trifugation at 10,000 xg for 20 min. The cells were washed twice with 10mM phosphate buffer (pH 7.0) and then suspened in 50 ml of the same buffer with 0.1% of various alkane derivatives. The mixtures were incubated at 30°C for 12 hr under continuous reciprocal shaking. The products were extracted with ethyl acetate then spotted on TLC plate to determine the character of the unknown compounds.

Instruments

A Hitachi gas chromatograph model 663-50 (Hitachi, Ltd. Tokyo, Japan) equipped with a flame ionization detector and containing a coiled Pyrex glass column (100cm×0.3cm) packed with 3% OV-17 coated on acid-washed, dimethylchlorosilane treated, 80/100 mesh Gaschron Q (Applied Science Laboratories) was used. The temperature of the column was 100°C. The temperature of the injector and detector was 200°C. The gas flow rates were 50ml/min for carriger gas N_2, H_2, and air. Gas chromatography-mass spectrometry (GC-MS) was carried out on a mass spectrometer Hitachi RMV-6 equipped with a jet separator. The conditions for the analysis were as follows: injection temperature, 200°C; oven temperature 100°C; source electron impact, 70eV; column packed with 2% OV-17 on 60/80.

Chemicals

All of the alkane derivatives used were purchased from Tokyo Kasei Kogyo Co., Inc. DAD was certified as 99.9% pure and the other alkane derivatives were all certified as above 95% pure.

RESULTS AND DISCUSSION

Isolation and Identification of Organisms

Microorgainsms utilizing DAD as carbon source were isolated from the soil using the enrichment technique. One of these, isolate DAD 2-3, was selected for this study. Isolate DAD 2-3 was identified as *Corynebacterium* (Lee, 1983).

Table 1. Assimilation of Various Alkane Derivatives

Alkane derivatives	Isolate DAD 2-3
1, 12-diaminododecane	good growth
Laurylamine	good growth in containing agar slant
Putrescine dihydrochloride	moderate growth
1-dodecanethiol	no growth
Thioanisole	no growth
1, 10-decanedithiol	no growth
1, 8-dicyanooctane	no growth
Laurylcyanide	no growth
1, 10-dichlorodecane	no growth
Dodecane	moderate growth

Growth Test on Various Alkane Derivatives

The results of the growth test are shown in Table 1. Dodecanethiol, Thioanisole, decanedithiol dicyanooctane, laurylcyanide and dichlorodecane were not utilized as the substrates by isolate DAD 2-3. The substrates such as DAD, putrescine dihydrochloride, dodecane and laurylamine (tested on agar slant) were utilized by isolate DAD 2-3. The growth of the organisms in these substrates was accompanied with the decreased in pH from 7.0 to 3.9~6.0.

The effect of adding emulgen to DAD medium on the growth of the organism is shown in

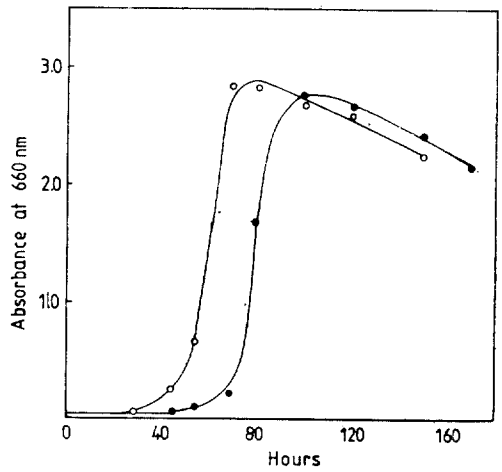

Fig. 1. Slightly accelerated growth of isolate DAD 2-3 due to emulgen.
○—○ : medium with emulgen
●—● : medium without emulgen

Fig 1. Isolate DAD 2-3 showed slightly accelerated growth.

Products from DAD

Several experiments were carried out to investigate the products formed by the microbial degradation of DAD.

The product formed by the isolate DAD 2-3 responded positively with bromphenol blue, 2, 4-dinitrophenylhydrazine, and triphenyltetrazolium indicating that it is acidic, it has carboxyl group and reducing properties, respectively. This compound was purified from the acidic fraction by column chromatography and analyzed by TLC, gas chromatography, and GC-MS. Based on the date of TLC (Fig. 2), the products were hydrolyzed and methylated for GC-MS. The mass spectrum of the methylated biological prouct was identical to that of synthetic α-ketoglutaric acid in its methylated form (Fig. 3). This biological product was identified as α-ketoglutaric acid by the results of TLC and GC-MS.

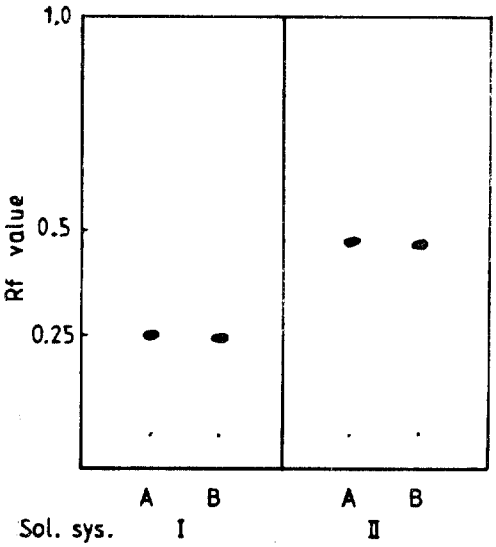

Fig. 2. Thin layer chromatography of products from DAD medium and α-ketoglutaric acid.
Sol. system I: benzene-1, 4 dioxane-acetic acid
Sol. system II: n-butanol-acetic acid-water
A : Products B : α-ketoglutaric acid

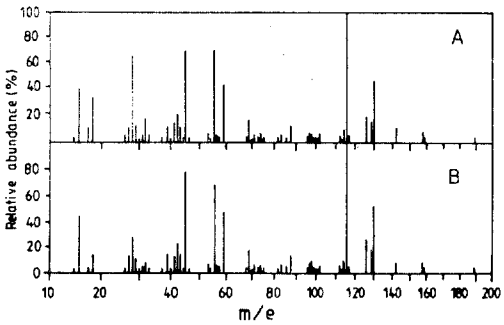

Fig. 3. Mass spectra of methylated biological products from isolate DAD 2-3 (A) and authentic α-ketoglutaric acid in its methylated form (B).

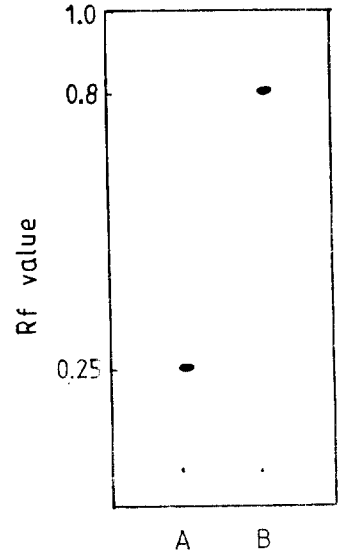

Fig. 4. Thin layer chromatography of acidic fraction from DAD medium without nitrogen source.
Sol. system: benzene-1, 4 dioxane-acetic acid
A : with nitrogen source
B : without nitrogen source

Isolate DAD 2-3 was tested to study the products formed in DAD medium without nitrogen source. The product formed by the isolate DAD 2-3 responded negatively with bromphenol blue, ninhydrin, and 2, 4-dinitrophenylhydrazine. The Rf value of the product was different from the Rf value of the product formed by isolate DAD 2-3 in the medium with NH_4NO_3 (Fig. 4). The results suggest that the products formed by the isolate in the medium without nitrogen source were different from that formed by the isolate in the medium with NH_4NO_3. The kind of DAD degradation products seemed

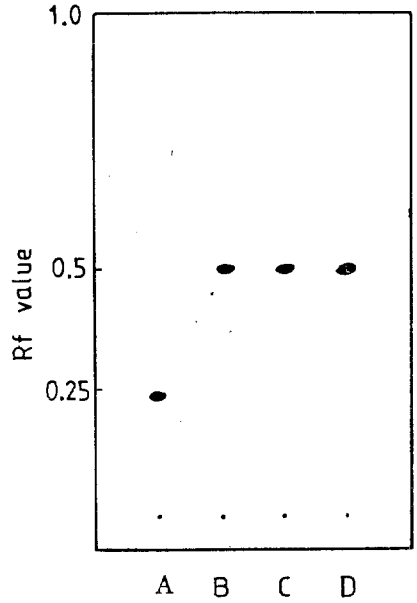

Fig. 5. Thin layer chromatography of acidic fraction from with glucose, putrescine and n-dodecane. Sol. system: benzene-1, 4 dioxane-acetic acid
A : products from DAD
B : acidic fraction from glucose
C : acidic fraction from putrescine
D : acidic fraction from n-dodecane

to be greatly affected by the nitrogen metabolism of the organism.

Products from Glucose, Putrescine and n-Dodecane

Isolate DAD 2-3 was tested to investigate the products from glucose, putrescine and n-dodecane in place of DAD as carbon source. TLC Rf values of these products were different position that formed by isolate DAD 2-3 in the medium with DAD as carbon source (Fig. 5). The products formed from glucose, putrescine and n-dodecane responded negatively with bromphenol blue. Therefore the products formed from glucose, putrescine and n-dodecane were different from that formed by isolate DAD 2-3 in the medium with DAD as carbon source. The results suggest the specificity of DAD as carbon source.

Products from Alkane Derivatives by Resting Cells

The products from various alkane derivatives were examined using the resting cells of isolate DAD 2-3. Rf values of all the products formed by isolate DAD 2-3 were different from that of α-ketoglutaric acid.

요 약

토양으로부터 diaminododecane 자화균 DAD2-3株를 분리하여 *Corynebacterium*속으로 동정하였다. DAD2-3株의 alkane유도체에 대한 생육특성조사에서 putrescine, dodecane, laurylamine 등은 탄소원으로 이용되었으나 dodecanethiol, thioanisole, decanedithiol, dicyanooctane, laurylcyanide, dichlorodecane등은 이용되지 못하였으며, emulgen 첨가에 의한 diaminododecane 자화촉진효과는 미세하였다.

DAD2-3株에 의해 diaminododecane 자화시 생성되는 중간생성물은 α-ketoglutaric acid로 동정되었다. 그러나 diaminododecane을 탄소원 뿐만 아니라 질소원으로도 이용하였을 경우 α-ketoglutaric acid와는 상이한 중간 생성물을 생성하였으며, 탄소원으로 diaminododecane 대신 glucose, putrescine, n-dodecane을 사용하였을 경우와 또한 resting cell을 이용하여 여러가지 다른 alkane유도체를 co-oxidation시키는 과정에서도 α-ketoglutaric acid와는 상이한 생성물이 생성되었다.

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