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Studies on Diaminododecane Utilization by Bacteria

(Part 1) Studies on Diaminododecane Utilization by *Corynebacterium* sp. DAD 2-2

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Diaminododecane 자화균에 관한 연구

(제 1 보) *Corynebacterium* sp. DAD 2-2의 diaminododecane 자화에 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 2-2, utilized DAD, putrescine dihydrochloride, dodecanethiol, dodecane and laurylamine. Thioanisole, decanedithiol, dicyanooctane, laurylcyanide, and dichlorodecane were not utilized. When emulgen 950 was added to the medium, the growth of DAD 2-2 was greatly accelerated. Isolated DAD 2-2 grown in the medium with DAD as carbon source formed ethyl α-ketoglutarate. Metabolic product of DAD 2-2 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 2-2 yielded products different from those they formed with DAD suggesting specificity of DAD as a carbon source.

Introduction

Studies on the transformation of biological organic compounds by microorganism have been widely undertaken recently. Only n-paraffin among non-biological organic compounds as substrate for microbial growth has been studied extensively (1). Many organic nitrogen compounds known to have strong inhibitory effect on the physiological activities of biological system. Among them amines have been known to induce cancer and malfunction in the liver. Many studies on the degradation of amines have been made (2-11) but little is known about

the degradation of long chain alkylamines. So far, only the degradation of long chain alkylamines has been reported (12).

This work was conducted with following aims:

a) to isolate and identify (up to genus level) DAD-utilizing organisms
b) identify the degradation products
c) examine the effect of carbon and nitrogen sources on the kind of degradation product.

Materials and Methods

Isolation and Identification of Microorganisms

Bacteria capable of utilizing DAD as carbon source were isolated from the soil by enrichment culture technique. The enrichment culture medium (DAD medium) 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; $MgSO_4 \cdot 7H_2O$, 0.01; $FeSO_4 \cdot 7H_2O$, 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. Fifty soil samples were used in the screening process. Soil samples (0.1 to 0.5g) were added to 8 ml medium in test tubes and incubated at 30°C in a reciprocal shaker for 7 days. The cultures (0.1 ml) were then transferred to a second enrichment liquid medium and incubated under the same conditions. From the tubes that showed visual growth, a loopful was streaked on nutrient agar plate. Each colony was transferred into a test tube containing 8 ml DAD medium and the flask was kept under the same conditions as above. A loopful of the culture that showed visual growth was again streaked on the DAD agar medium. The liquid culture-streaking process was repeated 5 times to obtain pure culture. The pure colonies on each plate were stored at 4°C and served as stock cultures.

Morphological and physiological characteristics of the isolated microorganisms were examined following the guides of Bergey's Manual of Determinative Bacteriology (13) and Komagata (14, 15). Meso-diaminopimelic acid was detected by TLC (16).

Growth Test

Growth test was carried out in test tubes each containing 10 ml 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 500 ml Sakaguchi flasks containing 100 ml of DAD medium and 25 ppm emulgen. Growth was estimated by measuring the absorbance at 660 nm 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 (Merck). 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 X g for 20 min was acidified to pH 2.0 and extracted as shown in Fig. 1. Extracts with ethyl acetate were concentrated using a rotary evaporator. The acidic fractions obtained were used for TLC.

For the purification and identification of products, the isolate DAD 2-2 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 x 25 cm, solvent system: cyclohexane-ethyl acetate-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, NMR, and GC-mass spectrometer.

In examining the effect of eliminating the nitrogen

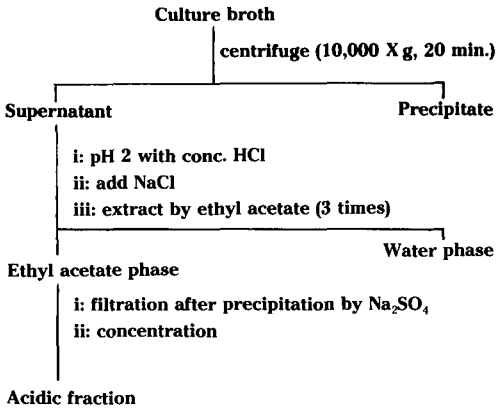

Fig. 1. Isolation of Acidic Fraction from the Culture Broth

source on the kind of degradation product, the isolate DAD 2-2 was grown in 100 ml DAD medium without NH_4NO_3 at 30°C under reciprocal shaking. Acadic 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 2-2 was grown to early stationary phase in 500 ml Sakaguchi flasks each containing 100 ml medium without DAD but with 0.1% glucose, putrescine, and n-dodecane. Acidic fractions formed by isolated DAD 2-2 were obtained as described in Fig. 1. The acidic fractions obtained were used for TLC.

Products from Alkane Derivatives

Cells grown to early stationary phase in 100 ml nutrient broth at 30°C were harvested by centrifugation at 10,000 X g for 20 min. The cells were washed thrice with 10 mM 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 (100 cm x 0.3 cm) 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. Infrared (IR) spectra were determined by a JASCO IR-S spectrometer equipped with NaCl prism using nujiol preparation technique. Nuclear magnetic resonance (NMR) spectra were performed on JEOL model JNM-FX 100 (Japan Electron Optics Laboratories, Tokyo) using 10 to 15 mg samples dissolved in 0.4 ml of $CDCl_3$. Tetramethylsilane was the internal reference. 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, 70 eV; column packed with 2% OV-17 on 60/80.

Chemicals

The chemicals used in the study are shown in Tables 1 and 2.

Table 1. Chemicals Used for the Growth Test

1,12-diaminododecane (DAD)	$H_2N(CH_2)_{12}NH_2$
Laurylamine (LA)	$CH_3(CH_2)_{11}NH_2$
Putrescine dihydrochloride (PD)	$H_2N(CH_2)_4NH_2 \cdot HCl$
1-dodecanethiol (DCT)	$CH_3(CH_2)_{11}SH$
Thioanisole (TH)	$C_6H_5SCH_3$
1,10-decanedithiol (DT)	$HS(CH_2)_{10}SH$
1,8-dicyanooctane (DC)	$NC(CH_2)_8CN$
Laurylcyanide (LC)	$CH_3(CH_2)_{11}CN$
1,10-dichlorodecane (DCD)	$ClCH_2(CH_2)_8CH_2Cl$
Dodecane (DD)	$CH_3(CH_2)_{10}CH_3$

Results and Discussion

Isolation and Identification of Organisms

Microorganisms utilizing DAD as carbon source were isolated from the soil using the enrichment technique. Isolate DAD 2-2 was identified as *Corynebacterium* (Table 3).

Table 2. Chemicals Used in the Resting Cell Experiment

1-chlorodecane (CD)	$CH_3(CH_3)_8CH_2Cl$
1,10-dichlorodecane (DCD)	$ClCH_2(CH_2)_8CH_2Cl$
Laurylcyanide (LC)	$CH_3(CH_2)_{11}CN$
1,8-dicyanooctane (DC)	$NC(CH_2)_8CN$
1-dodecanethiol (DCT)	$CH_3(CH_2)_{11}SH$
1,10-decanedithiol (DT)	$HS(CH_2)_{10}SH$
Cadaverine dihydrochloride (5CH)	$NH_2(CH_2)_5NH_2 \cdot 2HCl$
1,6-diaminohexane dihydrochloride (6CH)	$NH_2(CH_2)_6NH_2 \cdot 2HCl$
1,7-diaminoheptane (7CH)	$NH_2(CH_2)_7NH_2$
Octamethylenediamine (8CH)	$H_2N(CH_2)_8NH_2$
1,9-diaminononane (9CH)	$NH_2(CH_2)_9NH_2$
1,10-diaminodecane (10CH)	$NH_2CH_2(CH_2)_8CH_2NH_2$

Growth Test on Various Alkane Derivatives

The results of the growth test are shown in Table 4. Thioanisole, decanedithiol dicyanooctane, laurylcyanide and dichlorodecane were not utilized as the substrated by isolated DAD 2-2 tested. The substrates such as DAD, putrescine dihydrochloride, dodecanethiol, dodecane and laurylamine (tested on agar slant) were utilized by isolated DAD 2-2 tested. The growth of the organisms in these substrates was accompanied with the decreased in pH from 7.0 to 3.9-6.4.

The effect of adding emulgen to DAD medium on the growth of the organism is shown in Fig. 2. Isolate DAD 2-2 showed greatly accelerated growth.

Products from DAD

Several experiments were carried out to investigate the products formed by the microbial degradation of DAD. The results of TLC analysis (Fig. 3) indicate that DAD was degraded by the organism.

The product formed by the isolate DAD 2-2 showed one spot on TLC plate with Rf value of 0.5 (Fig. 3). This component 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, IR, gas chromatography, NMR, and GC-MS. Based on the date of IR (Fig. 4) and H-NMR (Fig. 5), the products were hydrolyzed and methylated for GC and GC-MS. The mass spectrum of the methylated biological product was identical to that of synthetic α-ketoglutaric acid in the methylated form (Fig. 6). This biological product was identified as ethyl α-ketoglutarate because this product had to be hydrolyzed first before it could be methylated.

Isolated DAD 2-2 was tested to study the products formed in DAD medium without nitrogen source. The product formed by the isolate DAD 2-2 responded negatively with bromphenol blue, ninhydrin, and 2,4-dinitrophenylhydrazine. The Rf value of the product

Table 3. Morphological, Cultural and Biochemical Characteristics of Isolate DAD 2-2.

Content	Isolate DAD 2-2
shape	slightly curved rods
cell size	0.6-0.8 by 3.0-4.0 μm
motility	non-motile
gram stain	strongly positive
spore	non-spore forming
acid-fast	not acid-fast
pleomorphism	not distinctive
type of cell division	snapping division
cell wall components	meso-diaminopimelic acid
colonies on nutrient agar	round, entire, convex, moist
colonies color	cream to gray
colonies surface	smooth surface
hydrolysis of gelatin	negative
hemolysis	positive
oxidation and fermentation	no action on carbohydrate
nitrate reduction	negative
methyl red test	negative
voges-proskauer	negative
indole test	negative
production of hydrogen sulfide	negative
lysine decarboxylase	positive
cytochrome oxidase test	negative
catalase test	positive
indole pyruvic acid	negative
urease	positive

Table 4. Assimilation of Various Alkane Derivatives

Alkane derivatives	Isolate DAD 2-2
DAD	good growth
LA	good growth in LA-containing agar slant
PD	good growth
DCT	moderate growth
TH	no growth
DT	no growth
DC	no growth
LC	no growth
DCD	no growth
DD	moderate growth

was different from the Rf value of the product formed by isolated DAD 2-2 in the medium with NH_4NO_3 (Fig. 7). The results suggest that the products formed by the isolated 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 to be greatly affected by the nitrogen metabolism of the organism.

Products from Glucose, Putrescine and n-Dodecane

Isolate DAD 2-2 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 same position that formed by isolated DAD2-2 in the medium with DAD as carbon source. But 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 2-2 in the medium with DAD as carbon source. The results suggest the specificity of DAD as carbon source.

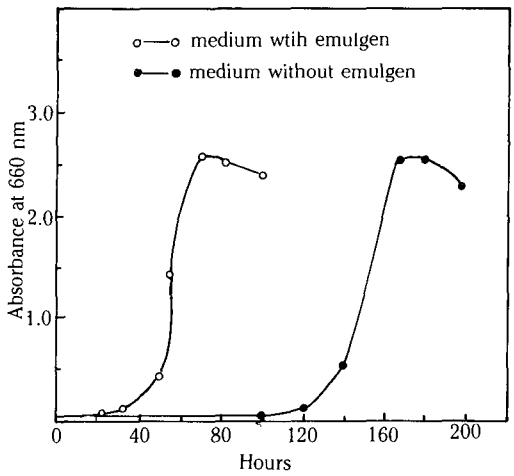

Fig. 2. Accelerated Growth of Isolated DAD 2-2 due to Emulgen

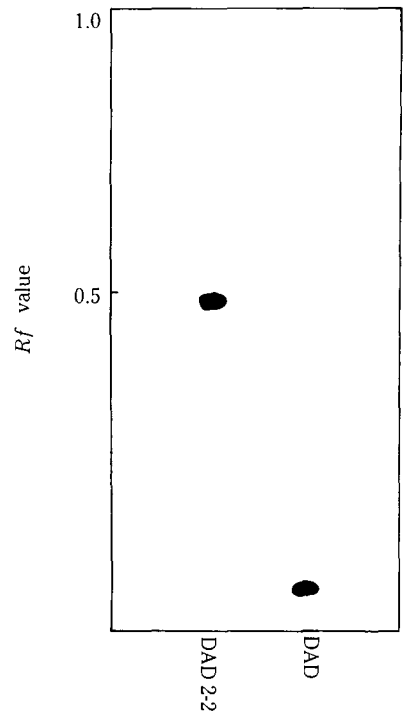

Fig. 3. Thin Layer Chromatography of Acidic Fraction from DAD Medium

Solvent system: benzene-1,4 dioxane-acetic acid

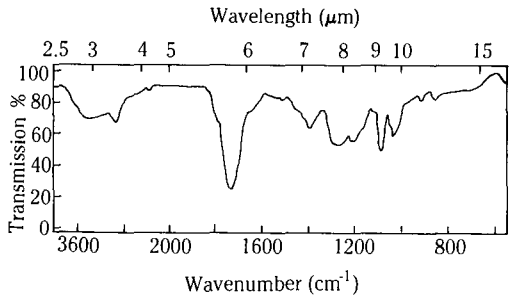

Fig. 4. Infrared Absorption Spectrum of Biological Product from Isolate DAD 2-2

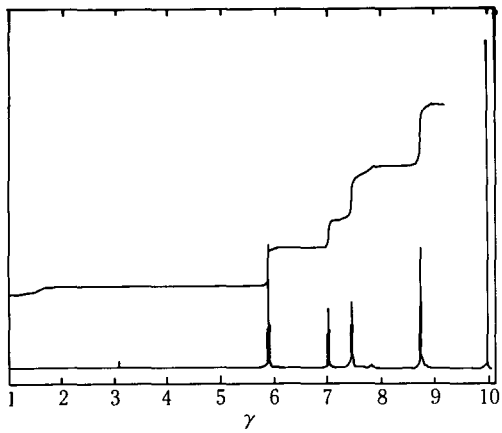

Fig. 5. NMR Spectrum or Biological Product from Isolate DAD 2-2

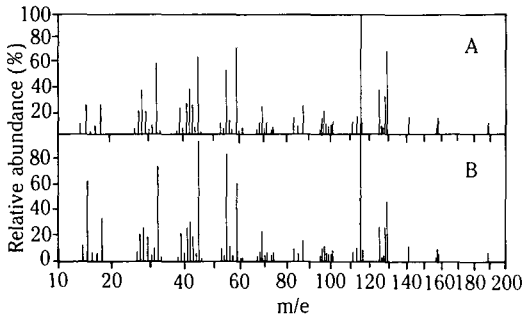

Fig. 6. Mass Spectra of Methylated Biological Product from Isolate DAD 2-2 (A) and Authentic α -Ketoglutaric Acid in its Methylated Form (B)

Products from Alkane Derivatives by Resting Cells

The products from various alkane derivatives (Table 1, 2) were examined using the resting cells of isolate DAD 2-2. Rf values of all the products formed by the isolated were different from that of ethyl α-ketoglutarate (Fig. 8).

요 약

토양으로 부터 diaminododecane 자화균 DAD 2—2株를 분리하여 검토한 결과 *Corynebacterium*속으로 동정되었으며 DAD2—2株의 alkane 유도체에 대한 생육특성조사에서 putrescine dihydrochloride, dodecanethiol, dodecane, laurylamine등은 탄소원으로 이용될 수 있었으나 thioanisole, decanedithiol, dicyanooctane, laurylcyanide, dichlorodecane등은 이용되지 못하였다.

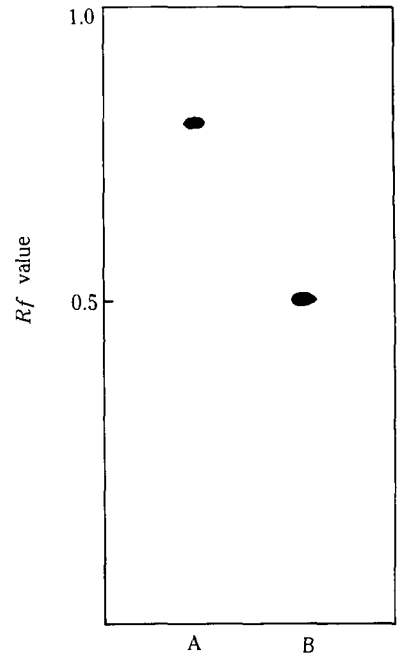

Fig. 7. Thin Layer Chromatography of Acidic Fraction from DAD Medium without Nitrogen Source

Solvent system: benzene-1,4 dioxane-acetic acid

A: with nitrogen source

B: without nitrogen source

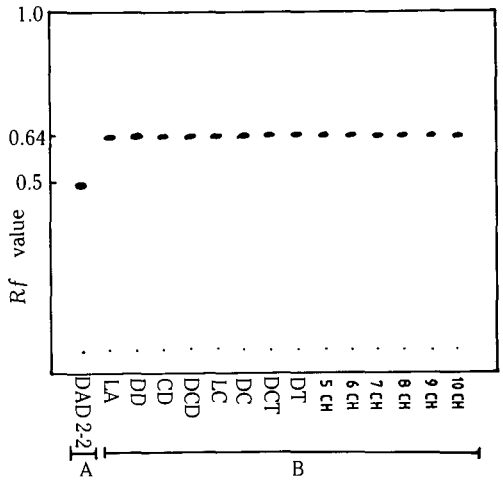

Fig. 8. Thin Layer Chromatography of Acidic Fraction from Medium with Alkane Derivatives by Isolate DAD 2-2

refer to Table 1 and Table 2 regarding abbreviations

A: products from DAD medium

B: acidic fraction from alkane derivatives

Emulgen 25ppm을 DAD배지에 첨가하였을 경우 DAD2—2株에 의한 diaminododecane 자화가 현저하게 촉진되었으며 diaminododecane자화시 생성되는 중간생성물은 ethyl α-ketoglutarate로 동정되었다. 그러나 diaminododecane을 탄소원뿐만 아니라 질소원으로도 이용하였을 경우에 있어서는 ethyl α-ketoglutarate와는 다른 생성물을 생성하였으며 diaminododecane 대신 glucose, putrescinc, n-dodecane을 생육기질로 이용하였을 때에도 ethyl α-ketoglutarate와는 다른 생성물을 생성하였다. 또한 resting cell을 이용하여 alkane 유도체를 co-oxidation시키는 과정에서도 ethyl α-ketoglutarate와는 상이한 생성물이 생성되었다.

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