Microbial Oxidation of Alkane Derivatives

(Part 1) Oxidation of Alkane Derivatives by *Corynebacterium* sp.

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Alkane 유도체의 미생물학적 산화

(제 1 보) *Corynebacterium* sp.에 의한 Alkane유도체의 산화

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ABSTRACT

Twelve Microorganisms capable of utilizing diaminododecane were isolated from the soil by enrichment culture technique. Seven strains of these were identified as *Corynebacterium*. The isolated strains were tested for the ability to utilize as carbon source, 10 different kind of alkane derivatives containing CN, NH_2, Cl, and SH groups. Laurylcyanide, dicyanooctane, chlorodecane, and dichlorodecane were not utilized by any of the isolated strains; putrescine dihydrochloride, cadaverine dihydrochloride, diaminododecane, and n-dodecane were utilized by all of the isolated strains; and all of the isolated strains except DAD 2-3 could utilize dodecylmercaptan. The alkane derivatives that did not serve as growth substrates were tested further in oxidation tests using resting cell preparation. Alkane derivatives that are being oxidized by all of the isolated strains are laurylcyanide and dichlorodecane. Dicyanooctane was also oxidized by all of the isolated strains except DAD 30L, chlorodecane was the only oxidized by the three isolated strains. The most remarkable substrate that is being oxidized is dichlorodecane containing CN groups diterminally. Evidence obtained with thin layer chromatography of ethyl acetate extracts of culture broth of isolated strains grown in some alkane derivatives shows that these alkane derivatives are degraded.

INTRODUCTION

Early work on microbial oxidations was carried out by microbiologists who were mainly interested in finding out whether alkane would support microbial growth. The discovery that hydrocarbons can be metabolized by microorganisms initiate a series of investigations which ultimately lead to different lines of developments (Kellio et al., 1965). Subsequent investigations not only elucidate the mechanisms of oxidation of various alkanes (Thijsse et al., 1958; Foster et al., 1963; Jones et al., 1968; pelz et al., 1973), but also suggest a great potential of microorganisms for the preparation of useful chemical intermediates and in the production of dietary supplements for human consumption (Fonken et. al., 1972).

Recently Omori and Alexander (1978) after an exhaustive survey of more than 500 soil enrichment cultures, succeeded in obtaining a strain of *Pseudomonas* sp. which was able to use dichlorononane as the sole source of carbon.

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Hydrocarbons containing one or more substituent chemical groups are chemically synthesized on large scale either for the use as potent herbicides and pesticides or for the exclusive use of research investigations.

Substituted hydrocarbons such as chlorinated pesticides and refrigerants are used widely and are already present in the natural environments in appreciable quantities. Even those used specifically in research investigations and in chemical synthesis may escape to the natural environment inadvertantly. Most of these substances are known to be toxic and irrespective of their origin, may pose a threat to animal and plant life in the natural environment unless the substituent groups are removed and the remainder of the molecule is available for intermediary metabolism by an appropriate organism in the natural environment. It is therefore worthwhile to study whether the microorganisms capable of oxidizing these chemicals are present in the natural environment and whether they are widely distributed in nature.

This report is concerned with the isolation and characterization of microorganisms capable of utilizing alkane derivatives. Evidence of the ability of microorganisms to oxidize these alkane derivatives is also presented.

MATERIALS AND METHODS

Isolation and Identification of Microorganisms

Bacteria capable of utilizing diaminododecane as their sole sources of carbon and energy were isolated from soil by enrichment culture technique. The enrichment culture medium had the following composition (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. Fifty samples were used to obtain the bacteria capable of utilizing the above substrates. Soil samples (0.1 to 0.5g) were added to 8ml 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 second enrichment liquid medium and incubated under the same condition. From the tubes that showed visual growth, a loopful was streaked on nutrient agar plate. Each colony was transferred into a test tube containing 8ml 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 cultured. The pure colonies on each plate were stored at 4°C and served as stock culture.

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

Growth Test

Each of the isolated organisms were tested for their ability to grow on the alkane derivatives as their sole sources of carbon and energy. The tests were carried out in 500ml shaking flask containing 100ml of the above medium with 0.2% alkane derivatives as the appropriate substrate. The substrates except dodecane were added to the sterilized medium without prior sterilization. Incubation was at 30°C under continuous reciprocal shaking and the growth was recorded visually up to seven days.

Preparation of Resting Cells

The nutrient broth medium was used for the preparation of resting cells. Cells grown to early stationary phase in 100ml nutrient broth at 30°C were harvested by centrifugation at 10,000×g for 20 min. The cells were washed thrice with 10mM phosphate buffer (pH 7.0). The cell paste was suspended in an appropriate amount of phosphate buffer and used for the assay of oxygen uptake (oxidation tests) stud-

ies by manometry.

Oxidation Tests

Oxidation tests were carried out using a conventional Warburgh's manometer. The amount of oxygen uptake by 0.5ml of cell suspensions was measured in the usual way. Substrates and enough amount of phosphate buffer solution to give a final volume of 2.7ml were placed in the main cup of the Warburgh flask and cell suspension in the side bulb. The center cup contained 0.2ml of 20% (w/v) aqueous potassium hydroxide solution. The temperature of the water bath was maintained at 30°C.

Isolation of Products

To obtain acidic products, the organisms were grown to early stationary phase in a 500ml shaking flasks each containing 100ml of the above medium with 0.2% of the appropriate substrate at 30°C with reciprocal shaking. The supernatant obtained by centrifugation at 10,000 ×g for 20min 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.

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.

Culture broth
centrifuge (10,000xg, 20min.)
Supernatant — Precipitate
i: pH 2 with conc. HCl
ii: add NaCl
iii: extract by ethyl acetate (3 times)
Ethyl acetate phase — Water phase
i: filtration after precipitation by Na_2SO_4
ii: concentration
Acidic fraction

Fig. 1. Isolation of acidic fraction from the culture broth

Chemicals

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

RESULTS AND DISCUSSION

Twelve microorganisms were isolated from the soil by enrichment technique. Seven strains (DAD 20L, DAD 2-2, DAD 2-3, DAD14L, DAD 1023L, DAD 181L, and DAD 30L) of these were selected for this investigation. The isolated organisms were characterized up to the genus level based on the methods, materials and diagnostic schemes given in the identification manuals (Williams and Wilkins Co., 1974; Komagata et al., 1969; 1970; 1972). All of seven isolated strains were identified as *Corynebacterium* (Table 1).

The results of the growth tests of the isolated strains are shown in Table 2. Laurylcyanide, dicyanooctane, chlorodecane, and dichlorodecane were substrates that were not utilized by any of the isolated strains tested. The substrates such as putrescine dihydrochloride, cadaverine dihydrochloride, diaminododecane and n-dodecane served as universal substrates for the majority of the isolated strains tested. All of the isolated strains except DAD 2-3 could utilize dodecylmercaptan. It is possible that diaminododecane utilizing bacteria are also able to degrade their substituted counterparts since

Table. 1. Morphological and biochemical characteristics of the DAD-utilizing bacteria.

Test	Isolated strains						
	DAD 20L	DAD 2-2	DAD 2-3	DAD 14L	DAD 1023L	DAD 181L	DAD 30L
Shape	rod	rod	rod	rod	rod	rod	rod
Type of cell division	sn	sn	sn	sn	sn	sn	sn
Cell wall composition	DAP	DAP	DAP	DAP	DAP	DAP	DAP
Gram staining	+	+	+	+	+	+	+
Motility	−	−	−	−	−	−	−
Spores	−	−	−	−	−	−	−
Oxidase (Kovacs)	−	−	−	−	−	−	−
Catalase	+	+	+	+	+	+	+
Oxidation & fermentation	−	−	−	−	−	−	−
Urease	+	+	−	+	+	+	+
Nitrate reduction	−	−	−	−	−	−	−
Methyl red	−	−	−	−	−	−	−
Voges Proskauer	−	−	−	−	−	−	−
IPA	−	−	−	−	−	−	−
Indole test	−	−	−	−	−	−	−
H_2S production	−	−	−	−	−	−	−
Lysine decarboxylase	+	+	+	+	+	+	+
Gelatin	−	−	−	−	−	−	−
Hemolysis	−	+	+	−	−	−	−
Acid-fast	−	−	−	−	−	−	−

DAP, meso-diaminopimelic acid; Sn, snapping division

Table 2. Assimilation tests on various alkane derivatives

Alkane derivatives	Isolated strains						
	DAD 20L	DAD 2-2	DAD 2-3	DAD 14L	DAD 1023L	DAD 181L	DAD 30L
Putrescine dihydrochloride	+	+	+	+	+	+	+
Cadaverine dihydrochloride	+	+	+	+	+	+	+
Diaminododecane	+	+	+	+	+	+	+
n-dodecane	+	+	+	+	+	+	+
Laurylcyanide	−	−	−	−	−	−	−
Dicyanooctane	−	−	−	−	−	−	−
Chlorodecane	−	−	−	−	−	−	−
Dichlorodecane	−	−	−	−	−	−	−
Dodecylmercaptan	+	+	−	+	+	+	+
1, 10 Decanedithiol	+	−	−	+	−	−	+

+, growth; −, no growth,

they are structurally similar to diaminododecane. During the growth of these isolated strains in their appropriate carbon sources the pH of the medium showed an appreciable change. The pH drop in most cultures were in the range of 3.9 ~6.0.

The alkane derivatives that did not serve as growth substrates were tested further in oxida-

Table 3. Oxidation tests on various alkane derivatives

Alkane derivatives	Isolated strains						
	DAD 20L	DAD 2-2	DAD 2-3	DAD 14L	DAD 1023L	DAD 181L	DAD 30L
Putrescine dihydrochloride	+	+	+	+	+	+	+
Cadaverine dihydrochloride	+	+	+	+	+	+	+
Diaminododecane	+	+	+	+	+	+	+
n-dodecane	+	+	+	+	+	+	+
Laurycyanide	0	0	0	0	0	0	0
Dicyanooctane	0	0	0	0	0	0	−
Chlorodecane	−	0	0	0	−	−	−
Dichlorodecane	0	0	0	0	0	0	0
Dodecylmercaptan	+	+	0	+	+	+	+
1, 10 Decanedithiol	+	0	0	+	−	−	+

+, growth as a sole source of carbon; 0, no growth, oxidation only; −, no growth, no oxidation.

tion tests using resting cell preparation of the relevant organisms. The resting cell preparation used for the oxidation tests were subjected to 30 min of starvation culture (buffer and cell suspension) to reduce the high endogenous activity. The concentration of the cell suspension used was adjusted to a common cell density by dilution with buffer when necessary. The relative rates of oxidation of different substrates varied from organism to organism. A glance at the Table 3 shows that many alkane derivatives which did not serve as growth substrates were oxidized rapidly by resting cell preparations. The alkane derivatives that is being oxidized under these condition by all of the isolated strains are laurylcyanide and dichlorodecane. Dicyanooctane was also oxidized by all of the isolated strains except strain DAD 30L, chlorodecane was the only oxidized by the three isolated strains (DAD 2-2, DAD 2-3 and DAD 14L). The isolate strain DAD 2-3 could not utilize dodecylmercaptan and decandithiol, but could oxidize dodecylmercaptan and decandithiol. The isolated strain DAD 2-2 could not utilize decanedithiol but could oxidize decanedithiol. The inability of these isolated strains to grow in the above substrates may be either due to their toxicity or to their incomplete metabolism by the organisms concerned. In most isolated strains tested the oxidation of substrates proceeded without any apparent lag. The most remarkable substrate that is being oxidized under these conditions is dichlorodecane containing CN groups diterminally.

Several experiments were carried out to in-

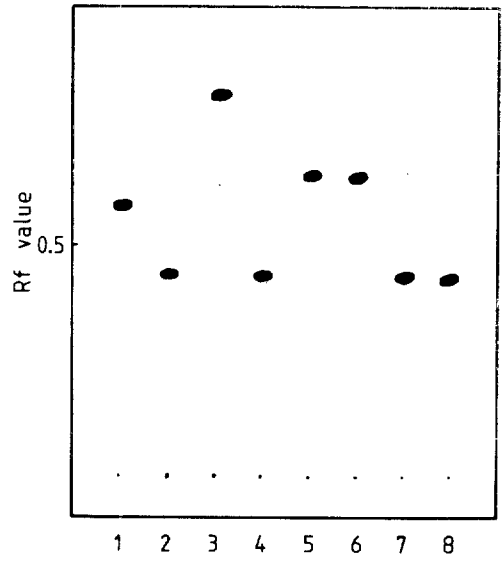

Fig. 2. Thin layer chromatography of acidic fraction from diaminododecane by isolated strains.
1. Substrate (diaminododecane)
2. Strain DAD 20L 3. Strain DAD 2-2
4. Strain DAD 2-3 5. Strain DAD 14L
6. Strain DAD 1023L 7. Strain DAD 181L
8. Strain DAD 30L

vestigate into the possible products formed during microbial degradation of diaminododecane. Preliminary results obtained by thin layer chromatography are shown Fig. 2. These evidence possibly indicates that diaminododecane were degraded by all of the isolated strains. The products formed by all the isolated strains resp onded positively with bromphenol blue, 2, 4-dinitrophenylhydrazine, and triphenyltetrazolium indicating that they are acidic, they have carboxyl group and reducing properties, respectively.

적 요

토양으로부터 diaminododecane 자화균 12株를 분리하였으며, 이들 분리균주 중 *Corynebacterium*속으로 동정된 7균주를 공시균으로 사용하였다. Cyan基, amine基, chlro基, thiol基등을 포함하고 있는 10가지의 alkane유도체들에 대한 이들 7균주의 생육특성조사에서 laurylcyanide. dicyanooctane, chlorodecane, dichlorodecane등은 탄소원의 생육기질로서 이용 될 수 없었으나, putrescine dihydrochloride, Cadaverine dihydrochloride, diaminododecane, n-dodecane 등은 생육기질로서 이용되었으며, dodecylmercaptan은 DAD 2-3株를 제외한 모든 분리균주에 의해 자화되었다.

Resting cell을 사용하여 생육기질로서 자화될 수 없는 alkane유도체에 대한 산화실험에서 laurylcyanide와 dichlorodecane은 모든 분리균주들에 의해 산화되었으며, dicyanooctane은 DAD 30L株를 제외한 모든 균주들에 의해 산화되었으나 chlorodecane은 단지 DAD 2—2株, DAD 2--3株, DAD 14L株등 만이 산화시킬 수 있었다. 모든 분리균주에 있어서 가장 현저한 oxygen uptake를 나타낸 alkane 유도체는 cyan基를 양말단에 가지고 있는 dichlorodecane이었다. 이들 분리균주들에 의해 diaminododecane으로부터 생성된 중간생성물은 대부분 서로 다른 Rf치를 나타내었다.

REFERENCES

1) Fonken G.S., and R.A. Johnson, 1972. Chemical Oxidations with Microorganisms.
2) Foster, J.W., and A.S. Kester, 1963. Diterminal Oxidation of Long-Chain Alkanes by Bacteria. J. Bacteriol. Vol. 85, 859-869.
3) Jones, D.F., and R. Howe, 1968. Microbiological Oxidation of Long-chain Aliphatic Compounds. Part 1. Alkanes and AlK-1-enes. J. Chem. Soc. (C), 2801~2808.
4) Kellio, R.E., and E.J. Mckenna, 1965. The Biology of Hydrocarbons. Ann. Rev. Microbiol., 19, 183.
5) Komagata, K., K. Yamada, and H. Ogawa, 1969. Taxonomic Studies on Coryneform Bacteria. I. Division of Bacterial cells. J. Gen.Appl. Microbiol., 15, 243.
6) Komagata, K., and K. Yamada, 1970. Taxonomic Studies on Coryneform Bacteria. II. Principal Amino Acids in the Cell Wall and Their Taxonomic Significance. J. Gen. Appl. Microbiol., 16, 103.
7) Komagata, K., and K. Yamada, 1972. Taxonomic Studies on Coryneform Bacteria. IV. Morphological, Cultural. Biochemical, and Physiological Characteristics. J. Gen. Appl. Microbiol., 18, 399.
8) Komagata, K., and K. Yamada, 1972. Taxonomic Studies on Coryneform Bacteria. V. Classification of Coryneform Bacteria, J. Gen. Appl. Microbiol., 18, 417.
9) Omori, T, and M. Alexander, 1978. Bacterial Dehalogenation of Haliogenated Alkanes and Fatty Acids. Appl. Environ. Microbiol., 35, 867 ~871.
10) Pelz, B.F., und H.J. Rehm, 1973. Terminale und subterminale Oxidation von n-Alkanen durch Schimmepilze., Arch. Mikrobiol, 92, 153~170.
11) Staneck, J.L., and G.D. Roberts, 1974. Simplified Approach to Identification of Aerobic Actinomycetes by Thin Layer Chromatography. Appl. Microbiol., 28, 226.
12) G.J.E. Thijsse, and A.C. Van der Linden, 1958. n-Alkane Oxidation by a *pseudomonas*. Studies on the Intermediate Metabolism. J. Microbiol. Serol., 24, 298.
13) Williams and Wilkins. Co., 1974. Bergey's Manual of Determinative Bacteriology. 8th. ed.,The Williams and Wilkins. Co., U.S.A.