Studies on Hydrocarbon-utilizing Microorganisms

Part I. Isolation, Identification and Characterization of Hydrocarbon-utilizing Bacteria

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For industrial utilization of hydrocarbon-assimilating bacteria and elucidation of mechanism of microbial hydrocarbon metabolism, the author isolated bacteria from oil-soaked soils subjecting to identification and characterization. Thirteen strains isolated were found to belong to Genus Pseudomonas, Alcaligenes, Acromobacter, Corynebacterium or Bacillus. These bacteria showed the capacity assimilating n-alkanes, but not aromatic hydrocarbons. In addition, some of these assimilated ethanol and others did diols but none of these decomposed polyvinylalcohol and a commercial neutral detergent.

Introduction

Recently, many studies on microorganisms utilizing hydrocarbon as a sole carbon source have been reported. In those studies, the microorganisms involved bacteria, yeasts and moulds and were able to utilize hydrocarbons such as aliphatic, olefinic or aromatic series.

In order to utilize hydrocarbon-assimilating microorganisms in commercial base, the following studies are carried out currently. (1) Production of single cell protein: The cells of bacteria or yeasts grown on media consisted of mineral salts and hydrocarbons were harvested to extract protein for food and fodder. The hydrocarbons used were natural gas[1], kerosene[2], gas oil[3] or other products in petroleum industry. The reports are aften found that the proteins from these microbial cells were rich in essential amino acids. (2) Production of amino acids: Mainly in this country, the studies were carried out on the production of amino acids such as glutamic acid[4], phenylalanine[5], ornithine, homoserine or valine from hydrocarbons such as n-alkanes, n-alkenes and hydrocarbon-mixtures in the culture broth. (3) Production of organic acids: It was reported that hydrocarbon-assimilating bacteria yielded in large quantity organic acids such as a α-ketoglutarate[6], citrate[7], fumarate[8] and dipicolinic acid[9]. (4) Production of vitamins and coenzymes: Carotenoids[10], vitamin B_6[11], vitamin B_{12}[12], biotin[13], coenzyme Q[14] and Cytochrome c[15] were produced by hydrocarbon-assimilating microorganisms from various hydrocarbons.

On the other hand, hydrocarbon-assimilating microorganisms were of physiological interest for microbiologist. Microbial metabolism of hydrocarbons has been studied by investigators. It was described the review by A. C Van der Linden and G. J. E Thijsse[16] that n-alkanes were oxidatively degradated to acetyl-CoA through β-oxidation pathway via the corresponding mono- or dibasic fatty acids and benzene was degradated to succinate and acetyl-CoA through catechol followed by 3-oxoadipate.

Hydrocarbon-utilizing microoganisms were widely distributed in nature. H. Iizuka et al[17]. identified and characterized the isolates from oil-brine, flooding-water, crude oil, cutting pieces,

drilling mud, bottom water of oil tank, sewage and soils obtained in petroleum zones in Japan.

Since we isolated hydrocabon-assimilating bacteria and moulds from bottom-water of jet fuel tank in order to elucidate the causes of contamination of jet fuel JP-4, we have taken an interest in these microorganisms and have isolated, identified and characterized the microorganisms from oil-soaked soils. Of these isolates, we found a bacterial culture producing red pigment in kerosene media and growing abundantly in naphthalene ones[13]. We determinated optimal cultural conditions for formation of this pigment, then purified and crystellized the pigment. The chemical structure is now under investigation.

In this paper, isolation, identification and characterization of hydrocarbon-assimilating bacteria from oil-soaked soils are reported.

Materials and Methods

(1) Isolation and Identification of Microorganisms.

Hydrocarbon-assimilating bacteria were isolated by enrichment culture method. After 1 g of oil-soaked soil was vigorously mixed with 10 ml of sterilized water in a test tube, 1 ml of the supernatant liquid was added, in L type test tube, to 10 ml of sterilized Medium A (Table 1) which was supplemented with 1 ml of kerosene sterilized by Seitz filter. The L type test tube was shaken on the Monod's type shaker for seveval days at 30°C. From the test tube in which microorganisms grew, one loopful culture broth was spread on kerosene agar plate and incubated for several days at 30°C. The colonies which appeared on the plate were transfered into 10 ml of the liquid media in L type test tube and incubated on shaker as described above. The microbial purity of the isolate was examined by plate culture of the broth in the test tube. When it was pure, the culture was transfered onto kerosene agar slant, and stocked. If the isolate was impure, enrichment culture were repeated till it was pure.

Table 1. The Compositions of Bassal Medium A.

$Na_2HPO_4 \cdot 12aq.$	0.3 (g)
KH_2PO_4	0.3
NaCl	0.05
$MgSO_4 \cdot 7aq.$	0.05
NH_4NO_3	0.5
Tap Water	100 ml
pH	7.0
Carbon Source	indicated in the text.

for plate and slant cultuers, 2 % agar was added.

Before identification, the isolates obtained were temporarily determinated with letter and number as is shown below (e. g. S1K1). S and K means soil sample and colony that emerged on the plate, respectively.

The diagnostic tests of each isolate were carried out according to "Manual of Microbiological Methods"[19], and identification was done according to "Bergy's Manual of Determinative Bacteriology"[20], 7th Ed., 1957.

(2) Cell Morphology and Gram-staining.

Diagnostic tests for cell shape, cell size, motility and Gram stain were carried out everyday with the cells cultivated in nutrient liquid media for 3 days and kerosene liquid media for 7 days. More details of morphology and flagella were observed with an electron-microscope. Gram stain was carried out by the method modified by Hucker.

(3) Cultural Characteristics.

The appearances of colonies on nutrient agar plate and of growth on nutrient agar slant were observed everyday, cultivating at 30°C for 7 days.

(4) Physiological Characteristics.

A) Reduction of nitrate: Peptone water broth containing 0.1 % KNO_3 was used for this test. After 2, 3, 4 and 5 day cultivation, nitrite was detected with α-naphthylamine and sulfanilic adid.

B) Production of hydrogen sulfide: Inoculated peptone water in a test tube which was equipped with a paper strip saturated with lead acetate solution was incubated for 7 days at 30°C or 37°C. Hydrogen sulfide produced was detected with color change of the strip to black.

C) Production of indole: Peptone water broth cultivated for 2, 3, 4 and 5 days at 30°C or 37°C was used for this test with Ehrlich-Böhmes' method.

D) Production of catalase: When one loopful cells from nutrient slant culture was transfered into 3 % H_2O_2 solution, continuous foam production for 30 minutes was cristerion for catalase positive.

E) Production of urease: One loopful cells from nutrient slant was transfered into 5 ml of 1 % urea solution with a small amount of toluene in test tube. After it was sealed, the test tube was incubated for 90 hours at 37°C. Ammonia was detected with Nessler's reagent.

F) Production of amylase: After cultivation on nutrient agar plate containing 2 % soluble starch for 3 days, the presence of amylase was detected by pouring dilute iode solution onto the plate.

G) Acid production in milk: Litmus milk culture was used for this test. If litmus indicator became red for 2 weeks, acids were produced.

H) Rennet production: If milk was coagulated without formation of acid, the result was positive.

(5) Cleavage of Sugars.

A) Assimilation of sugars: After the bacteria were inoculated in Medium A containing 1 % sugar instead of kerosene, the growth was observed everyday for one week at 30°C.

B) Production of acids: Peptone water culture broth containing 0.002 % B. T. B indicator and 0.5 % carbohydrates was used for this test. The production of acids made the indicator yellow.

C) Production of gas: Semisolid agar stab (1 % agar-containing nutrients supplemented with 1 % sugar.) was used for this test.

(6) Presence of Endospore.

The culture heated to 85°C for 10 minutes was transfered to nutrient medium in a test tube, then being cultivated at 30°C for several days. Growth of bacteria shows endospore formation positive.

(7) Utilization of Various Other Substances.

This test was carried out by cultivation of microorganisms in the Medium A containing substances tested instead of kerosene in L type test tube on shaker for one week at 30°C.

Results and Discussions

Thirteen strains of bacteria were isolated from oil-soaked soils being identified and characterized as follows. The results are presented in Table 2 to 13 and Figure 1 to 4.

(1) Gram-Negative Strains Producing Non-Spores.

A) Strains possessing polar flagella; S34K1, S40K2 and S40K3.

The cells of these bacteria were rod-shaped, having polar flagella. According to the descriptions of "Bergy's Manual", of such bacteria, those which does not contain photosynthetic pigment belong to Family Pseudomonadaceae in Order Pseudomonadales. Since our strains were not attached to substrate and were heterotrophic and attacked oxidatively glucose and other sugars, the strains were considered to belong to Genus Pseudomonas in this Family. Many members in this Genus produce a water-soluble pigment which is bluish, greenish or brownish in color. But our strains produced no water-soluble pigment.

(a) S34K1 (Figure 1)

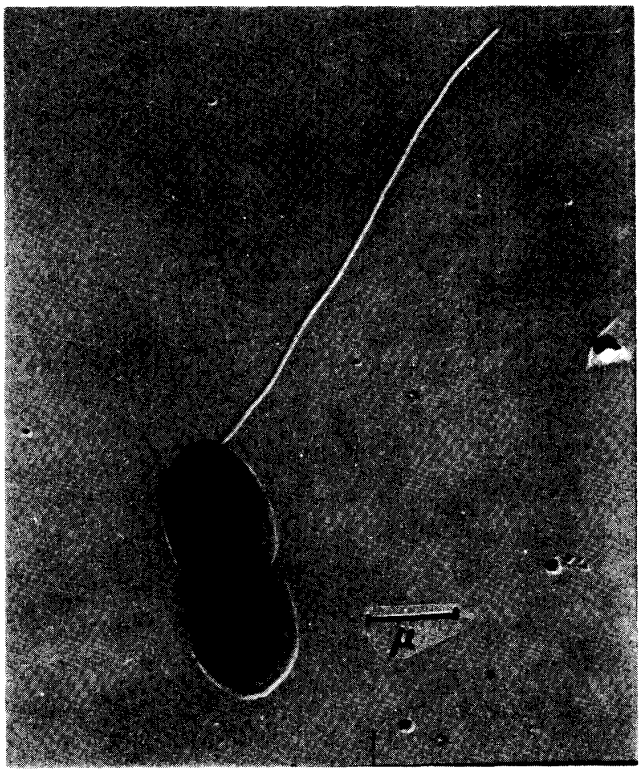

Figure 1 Polar flagella of Strain S34K1. ×1,0000

This strain showed similar characteristics to Pseudomonas salopia. Therefore, S34K1 was identified as the species. According to the "Bergy's Manual", the description of the species is as follows.

Pseudomonas salopia Gray and Thornton, 1928. Shape: Rods, 0.7 to 1.0 by 1.1 to 3.0 microns, singly and in pairs. One to six polar flagella. Gelatin stab: No liquefaction. Agar colonies: Circular or amoeboid, white to buff, flat to convex, smooth, glistening, translucent border, entire. Agar slant: Filiform, whitish, raised, smooth, glistening, lobate. Broth: Turbid with pellicle. Nitrite: Not produced from nitrate. Starch: Not hydrolyzed. Acid:

Table. 2 Cell Morphology and Cell Stain.

	Gram stain	Cell Shape	Cell Size. (microns)	Flagella.
S30K1	+	Long rods, singly or in pairs.	0.5 to 0.8 by 3.8 to 6.0	Non.
S31K1	+	Long rods, formed variably.	0.5 by 3.5 to 4.0	Non.
S31K2	+	Curved rods, singly or in pairs.	0.6 to 0.7 by 4.0 to 8.0	Non.
S34K1	−	Rods, singly or in pairs.	1.2 by 2.1 to 2.5	Polar.
S37K1	+	Long rods, singly or in pairs.	0.4 to 0.6 by 4.5 to 6.5	Non.
S40K1	−	Rods, singly or in pairs.	0.8 to 1.0 by 1.5 to 2.0	Polar.
S40K2	−	Rods, singly or in pairs.	0.6 to 0.8 by 1.5 to 1.8	Polar.
S40K3	−	Rods, singly or in pairs.	1.0 to 1.3 by 2.0 to 3.0	Polar.
S40K4	−	Rods, singly or in pairs.	1.2 to 1.3 by 2.5 to 2.7	Peritrichous
S41K2	−	Rods, singly or in pairs.	0.9 to 1.2 by 2.0 to 3.0	Peritrichous
S42K1	−	Rods, singly or in pairs.	1.0 by 1.8 to 2.3	Peritrichous
S42K2	+	Long rods branched.	0.4 to 0.5 by 3.2 to 6.0	Non.
S43K1	+	Long rods branched.	0.5 to 0.7 by 3.0 to 5.5	Non.

Table. 3 Characteristics of Growth on Nutrient Agar.

	Colonies on the plate.				Growth on the Slant.		
	Shape.	Color.	Rising.	Margin	Growth.	Surface.	Color.
S30K1	Circular.	Nacreous.	Pulvinate.	Entire.	Abundant.	Smooth.	Opaque.
S31K1	Filamentous.	Cretacous.	Umbonate.	Erose	Abundant.	Filiform.	Dull.
S31K2	Circular.	Nacreou.	Pulvinate.	Entire.	Abundant.	Filiform.	Opaque.
S34K1	Circular.	Butylous.	Convex.	Entire.	Moderate.	Smooth.	Tranpsarent.
S37K1	Puntiform.	Sebaceouce.	Raised.	Entire.	Abundant.	Smooth.	Opaque.
S40K1	Circular.	Sebaceous.	Pulvinate.	Entire.	Abundant.	Filiform.	Dull.
S40K2	Filamentous.	Sebaceous.	Umbonate.	Erose.	Moderate.	Smooth.	Transparent.
S40K3	Circular.	Nacreous.	Umbonate.	Entire.	Moderate.	Smooth.	Transparent.
S40K4	Circular.	Sebaceous.	Umbonaite.	Entire.	Moderate.	Smooth.	Transparent.
S41K2	Circular.	Oleaginous.	Convex.	Entire.	Abundant.	Smooth.	Translucent.
S42K1	Filamentous.	Sebaceous.	Pulvinate.	Erose.	Abundant.	Smooth.	Translucent.
S42K2	Irregular.	Nacreous.	Umbonate.	Undulate.	Abundant.	Smooth.	Translucent.
S43K1	Circular.	Nacreous.	Pulvinate.	Entire.	Abundant.	Filiform.	Dull.

Table. 4 Appearences of Growth in Nutrient Broth and on Potato.

	In nutrient broth			On potato		
	Surface	Turbid	Sediment	Growth	Color	
S30K1	Membranous	Moderate	Compact	Moderate	Pink	Glistening
S31K1	Membranous	Slight	Flocculent	Moderate	Dark orange	Dull
S31K2	Membranous	Moderate	Compact	Moderate	Pink	Glistening
S34K1	Pellicle	Strong	Compact	Moderate	Brown	Glistening
S37K1	Pellicle	Strong	Viscid	Moderate	Pink	Glistening
S40K1	Pellicle	Slight	Viscid	Moderate	Gray	Dull
S40K2	Pellicle	Strong	Viscid	Moderate	Brown	Glistening
S40K3	Membranous	Strong	Visce	Moderate	White	Glistening
S40K4	Pellicle	Strong	Viscid	Moderate	Yellow	Glistening
S41K2	Membranous	Strong	Flocculent	Moderate	Yellow	Glistening
S42K1	Membranous	Strong	Viscid	Moderate	Brown	Glistening
S42K2	Membranous	Strong	Compact	Moderate	Pink	Glistering
S43K1	Membranous	Slight	Flocculent	Moderate	Orange	Dull

Table. 5 Growth at 37°C and 42°C, Heat-Resistance at 85°C. and Growth in 7% NaCl Broth.

	Growth at 37°C	Growth at 42°C	Resistance to 85°C	Growth in 7% NaCl broth.
S30K1	−	−	−	−
S31K1	−	−	±	−
S31K2	+	−	−	−
S34K1	+	±	−	−
S37K1	−	−	−	−
S40K1	+	−	−	−
S40K2	+	−	−	−
S40K3	+	+	−	−
S40K4	+	+	−	−
S41K2	+	−	±	−
S42K1	+	−	−	−
S42K2	+	−	+	−
S43K1	−	−	−	−

Table. 6 Physiological Tests. (1)

	Action on litmus milk.	Action on gelatin stab.
S30K1	Alkaline. Reduced. Orange sediment.	Slight growth. Non-liquefaction.
S31K1	Alkaline. Reduced. Orange sediment.	Slight growth. Non-liquefaction.
S31K2	Alkaline. Reduced.	Moderate growth. Non-liquefaction.
S34K1	Alkaline.	Moderate growth. Non-liquefaction.
S37K1	Alkaline.	Slight growth. Non-liquefaction.
S40K1	Pink to yellow. Unchanged.	Slight growth. Non-liquefaction.
S40K2	Unchanged.	Moderate growth. Non-liquefaction.
S40K3	Acid. Coagulated. Pink sediment.	Stratiform liquefaction.
S40K4	Unchanged. Coagulated.	Stratiform liquefaction.
S41K2	Alkaline. Coagulated.	Crateriform liquefaction.
S42K1	Alkaline.	Moderate growth. Non-liquefaction.
S42K2	Unchanged.	Moderate growth. Non-liquefaction.
S43K1	Alkaline. Pink sediment.	Slight growth. Non-liquefaction.

Table. 7 Physiological Tests. (2)

	Production of Nitrate	Production of Indole.	Production of H_2S.	Production of Urease.	Production of Catalase.	Production of Amylase	Growth at 37°C.
S30K1	+	−	+	+	+	−	−
S31K1	+	−	−	−	+	−	−
S31K2	−	−	+	+	++	−	+
S34K1	−	−	−	−	+	−	+
S37K1	+	−	+	+	++	−	−
S40K1	++	+	+	+	+	−	+
S40K2	+	−	−	−	+	−	+
S40K3	−	−	−	−	+	−	+
S40K4	−	−	−	−	+	−	+
S41K2	+	−	−	−	+	−	+
S42K1	+	−	−	++	+	−	+
S42K2	++	−	++	++	+	−	−
S43K1	+	−	++	++	++	−	−

Table. 8 Utilization of Carbohydrates.

	Sucrose	Glucose	Lactose	Galactose	Maltose	Mannose	Fructose
S30K1	−	−	−	−	−	−	−
S31K1	−	−	−	−	−	−	−
S31K2	−	−	−	−	−	−	−
S34K1	−	++	−	+	−	+	−
S37K1	−	−	−	−	−	+	−
S40K1	+	+	+	+	−	+	−
S40K2	−	++	−	+	−	+	+
S40K3	−	+	−	+	−	+	+
S40K4	−	+	−	−	−	−	−
S41K2	−	+	−	+	−	−	+
S42K1	+	+	−	+	−	−	−
S42K2	+	+	−	+	−	+	+
S43K1	+	+	−	+	−	+	−

Symbol; − : Not utilized, + : Utilized, ++ : Strongly utilized.

Table. 9 Production of Acids from Carbohyrates.

	Glucose.	Sucrose.	Maltose.	Lactose.	Xylose.	Dextrin.	Starch.	Glycerol.
S30K1	−	−	−	−	−	−	−	−
S31K1	−	−	−	−	−	−	−	−
S31K2	±	−	−	−	−	−	−	−
S34K1	+	−	−	−	−	−	−	−
S37K1	−	−	−	−	−	−	−	−
S40K1	+	+	−	−	+	±	−	−
S40K2	−	−	−	−	−	−	−	−
S40K3	−	−	−	−	−	−	−	−
S40K4	−	−	−	−	−	−	−	−
S41K2	−	−	−	−	−	−	−	−
S42K1	−	−	−	−	−	−	−	−
S42K2	−	−	−	−	−	−	−	−
S43K1	−	−	−	−	−	−	−	−

− : Not produced, ± : Slightly produced, + : Produced

Table. 10 Utilization of Mono-and Dibasic Acids.

	Monobasic Acids.				Dibasic Acids.			
	Formate.	Acetate.	Caproate.	Palmitate.	Succinate.	Glutarate.	Adipate.	Citrate.
S30 K1	−	−	−	+	−	−	−	−
S31 K1	−	−	−	−	−	−	−	−
S31 K2	−	−	−	−	±	−	−	−
S34 K1	±	−	++	+	++	++	−	++
S37 K1	−	−	−	−	−	−	−	−
S40 K1	−	−	−	+	++	+	++	++
S40 K2	−	−	−	+	++	+	++	++
S40 K3	−	+	−	+	++	++	++	++
S40 K4	−	++	−	+	++	++	−	++
S41 K2	−	−	−	+	++	++	++	++
S42 K1	±	++	−	+	++	+	++	++
S42 K2	−	−	±	+	++	+	++	++
S43 K1	−	++	−	+	+	+	++	++

Symbol ; − : Not utilized, ± : Slightly utilized, + : Utilized, ++ : Strongly utilized.

Table. 11 Utilization of n-Alkanes.

	Nonane.	Decane.	Undecane.	Dodecane.	Tridecane.	Tetradecane.	Hexadecane.	Octadecane.
S30 K1	+	++	++	++	++	++	−	−
S31 K1	+	+	++	++	+	−	++	++
S31 K2	+	++	+	++	++	−	++	++
S34 K1	+	+	++	+	−	−	−	−
S37 K1	+	−	−	+	+	−	+	+
S40 K1	−	+	−	+	−	−	−	−
S40 K2	−	+	+	−	−	−	−	−
S40 K3	++	++	++	++	+	+	+	+
S40 K4	++	++	++	−	−	−	−	−
S41 K2	−	−	+	+	−	+	−	−
S42 K1	++	++	++	++	++	+	++	++
S42 K2	−	+	++	++	++	−	++	++
S43 K1	−	+	++	++	++	+	++	++

Symbol ; − : Not utilized, + : Utilized, ++ : Strongly utilized.

Table. 12 Uuilization of n-Alkenes and Aromatics.

	n-Alkenes.			Aromatics.				
	Octene.	Decene.	Dodecene.	Benzene.	Toluene.	Cresol.	Naphthalene.	Anthracene.
S30 K1	−	+	−	−	−	−	−	−
S31 K1	−	−	−	−	−	−	−	−
S31 K2	−	−	−	−	−	−	−	−
S34 K1	−	−	−	−	−	−	−	−
S37 K1	−	+	−	−	−	−	−	−
S40 K1	−	+	−	−	−	−	−	−
S40 K2	−	−	−	−	−	−	−	−
S40 K3	+	+	−	−	−	−	+	−
S40 K4	−	−	++	−	−	±	−	−
S41 K2	−	+	−	−	−	−	−	−
S42 K1	−	+	+	−	−	−	−	−
S42 K2	−	+	−	−	−	−	−	−
S43 K1	−	±	−	−	−	−	−	−

Symbol; − : Not utilized, ± : Slightly utilized, + : Utilized.

Table. 13 Utilization of Alcohols and Others.

	Methanol.	Ethanol.	1, 4-Butanediol.	1, 3-Butanediol.	1, 2-Propanediol.	P. V. A *	Neutral** detergent.	Acetone.
S30 K1	−	+	−	−	−	±	−	−
S31 K1	−	−	−	−	−	−	−	−
S31 K2	−	NT	−	−	−	−	−	−
S34 K1	−	+	−	++	++	−	−	±
S37 K1	−	NT	−	−	−	−	−	−
S40K1	−	−	−	−	+	−	−	−
S40 K2	−	−	−	−	−	−	−	−
S40 K3	−	++	−	++	++	−	−	+
S40 K4	−	++	++	++	++	−	−	−
S41 K2	−	−	−	++	++	−	−	−
S42 K1	−	++	++	−	++	−	−	−
S42 K2	−	++	±	−	−	−	−	−
S43 K1	−	++	±	−	+	±	−	±

Symbol; NT: Not tested, − : Not utilized, ± : Slightly utilized, + : Utilized, ++ : Strongly utilized

* P. V. A: Polyvinylalcohol ($\bar{P}=500$)

** Neutral detergent: Soft type Neutral detergent (Lion Yushi K. K.)

Produced from glucose and sucrose.

(b) S40K2

This strain was similar to Pseudomonas oleovorans, except not hyhrolyzing starch. Then this strain could be regarded as a variant of Pseudomonas oleovorans.

Pseudomonas oleovorans Lee and Chandler, 1941. Shape: Short rods, 0.5 by 0.8 to 1.5 microns, occurring singly and in pairs. Gelatin stab: No liquefaction. Agar colonies: Smooth, convex, shiny, opaque, creamy, fluorescent by transmitted light. Edge entire in young colonies. Agar slant: Growth raised, smooth, fluorescent, edge erose. Litmus milk: No liquefaction. Indole: Not produced. Potato: Good growth. Nitrite: Produced. Starch: Hydrolyzed. Acid from sugars: Not produced.

(c) S40K3

This strain could be considered to be a variant of Pseudomonas pseudonallei, for this strain did not produce acids from carbohydrates.

Pseudomonas pseudomallei (Whitmore, 1913) Hayhes, comb. nov. Shape: Short rods, with rounded ends, occurring singly and in short chains. Possess 1 to 4 polar flagella. Gelatin stab: Moderate, crateriform liquefaction. Agar colonies: Circular, slightly raised, opaque, cream-colored with irregular margin. Broth: Turbid with pellicle. Litmus milk: Curling with slowly developed acidity; pink sediment; may be digested. Potato: Vigorous, cream-colored growth.

Indole: Not produced. Acid: produced from glucose, maltose, lactose, sucrose and mannitol.

B) Strains possessing peritrichous flagella: S40K1, S40K4, S41K2 and S42K1.

These strains were rod-shaped, motile by means of peritrichous flagella and non-chromogenic to yellow or orange on ordinary agar media. According to "Bergy's Manual", such bacteria belong to Family Achromobacteriaceae in Order Eubactriales. There are five genera in this Family: Alcaligenes, Achromobacter, Flavobacterium, Agarbacterium and Beneckea. Since our strains neither attacked agar, nor produced acids from sugars, in addition to non-chromogenesis on nutrient agar, they were assigned to either Alcaligenes or Achromobacter.

(a) S41K2

This strain were motile, liquefied gelatin and peptonized milk. Therefore, this could be assigned to Alcaligenes bookerii. However, the strain S41K2 reduced nitrate to nitrite, while Achromobacter bookerii does not. Thus, the strain S41K2 might be classified as a variant of Alcaligenes bookerii.

Alcaligenes bookerii Bergy et al., 1923. Shape: Rods. 0.5 by 1.5 to 2.0 microns, occurring singly. Motile by means of peritrichous flagella. Gram-negative. Gelatin stab: slow, saccate liquefaction, becoming stratiform. Agar colonies: Thin, transparent, with opaque center and indistinct margin. Broth: Turbid, with viscid sediment. No pellicle. Litmus milk: Alkaline. Soft curd. Litmus reduced. Peptonization. Potato: Luxuriant, yellowish white, moist growth. Medium is darkened. Indole: Not produced. Acid: Not produced carbohydrates. Nitrite: Not produced.

(b) S42K1

This strain was similar to Alcaligenes faecalis, but did not produce urease, though Alcaligenes faecalis does.

Alcaligenes faecalis Castellani and Chalmers, 1919. Shape: Rods, 0.5 by 1.0 to 2.0 microns, occurring singly, in pairs and in chains. Motile by means of peritrichous flagella. Gram-negative. Gelatin stab: Gray surface growth. No liquefaction. Agar colonies: Opaque, entire, non-chromogenic. Agar slant: White, glistening, non-chromogenic. Litmus milk: Alkaline. Potato: Scant to abundant, yellowish to browish growth. Indole: Not produced. Hydrogen sulfide: Not produced. Acid: Produced from carbohydrates slightly, if at all.

(c) S40K1

The stain S40K1 liquefied gelatin, unchanged milk, produced nitrite from nitrate and produced acid from glucose, so the strain might be identified as Achromobacter isophagus.

Achromobacter isophagus Bergy et al., 1930. Shape: Rods, 0.8 to 1.0 by 1.0 to 5.0 microns. Motile by means of peritrichous flagella. Gram-negative. Gelatin stab: Liquefaction. Agar colonies: Circular or amoeboid, whitish, flat, raised, smooth, translucent, entire. Agar slant: Filiform, white to buff, flat, undulate. Broth: Turbid. Litmus milk: Unchanged. Acid: Produced from glucose and sucrose. Starch: Hydrolyzed. Nitrite: Produced.

(d) S40K4 (Figure 2)

The strain S40K4 was identified as a variant of Achromobacter liquefaciens. It could grow at 37°C, though Achromobacter liquefaciens cannot.

Achromobacter liquefaciens Bergy et al., 1923. Shape: Short, rather thick rods with rounded ends. Occur singly. Actively motile. Possess peritrichous flagella. Gram-negative. Gelatin stab: Napifrom liquefaction. Agar slant: Dirty white, Spreading growth. Broth: Turbid. Litmus milk: Unchanged. Potato: Light yellow growth. Indole: Not produced. Nitrite:

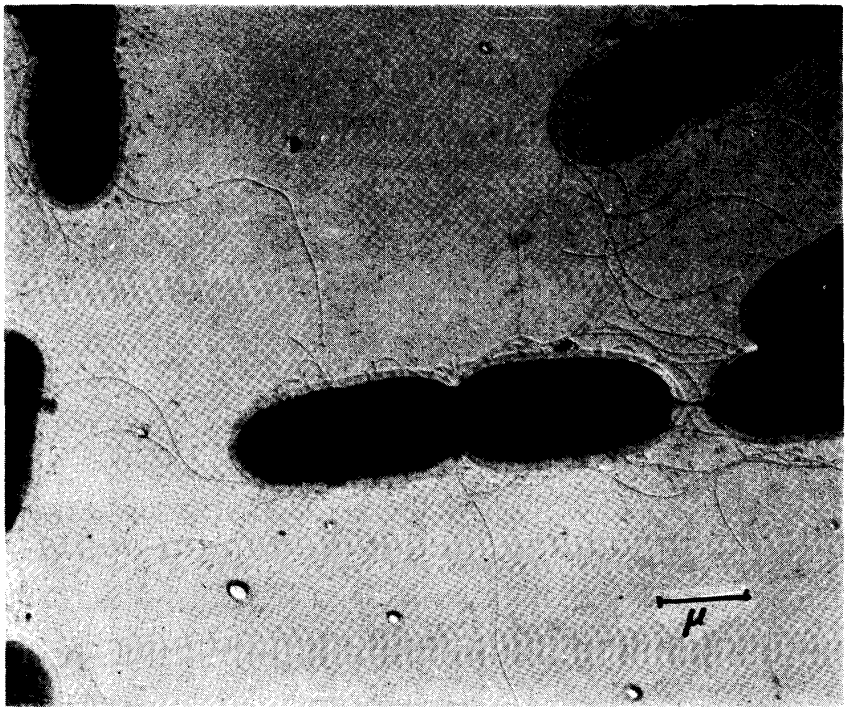

Figure 2 Peritrichous flagella of Strain S40K4. × 10,000

Not produced from nitrate. Does not grow at 37°C.

(2) Gram-Positive Strains Producing Non-Spores.

S30K1, S31K1, S31K2, S37K1 and S43K1 (Figure 4).

All of these strains were Gram-positive, non-motile, branching or straight rod-shaped and rich in granule. According to the description of "Bergy's Manual", the bacteria showing such morphology belong to Family Corynebacteriaceae in Order Eubacteriales. Of these strains, S31K2, (Figure 3) showed negative reaction in nitrate reduction, indole production and acid

Figure 3 Granule-rich cells of Strain S31K12. × 5, 000

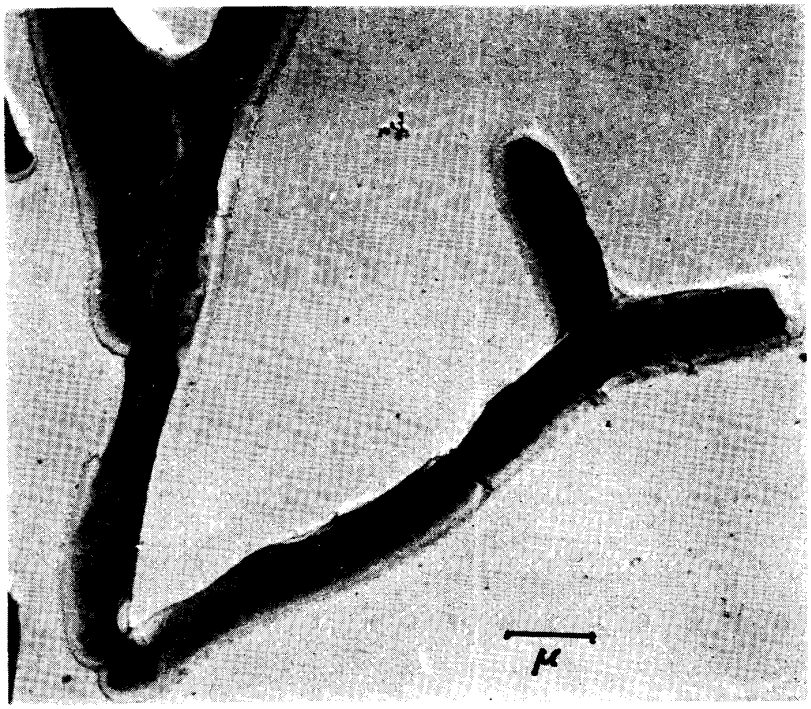

Figure 4 Branched cells of Strain S43K1. × 10, 000

formation from carbohydrate, and positive one in alkalifying litmus milk. Thus, this strain was identified as Corynebacterium bovis, though a little difference was observed in the appearance of growth on potato plug.

Other strains showed positive reaction in nitrate reduction, production of hydrogen sulfide and hydrolyzation of starch, and negative one in indole formation and gelatin liquefaction. Moreover, these strains yielded tan, yellow or pink growth on potato. In "Bergy's Manual", the species having such characteristics are described as Corynebacterium equi. Then, three strains could be identified as Corynebacterium equi. On the other hand, S31K1 was not identified as any of the species in Corynebacterium, as it was incapable of producing hydrogen sulfide.

Corynebacterium bovis Bergy et al., 1923. Shape : Slender rods, 0.5 to 0.7 by 2.5 to 3.0 microns, which are barred and clubbed. Non-motile, Gram-positive. Gelatin stab : Slight, gray, flat surface growth. Agar colonies : Circular, gray, slightly raised, radiate, undulate, dry. Agar slant : Thin, gray, filiform, dry growth.

Broth : Slight, granular sediment. Litmus milk : Slowly becomes deeply alkaline. Potato : No growth. Indole : Not produced. Acid : Not produced carbohydrate. Nitrite : Not produced.

Corynebacterium equi Magnusson, 1923. Shape : Rods variable according to medium. Non-motile. Gram-negative. Gelatin stab : Good growth. No liquefaction. Agar colonies : Usually moist, smooth and glistening, tan to yellow or pink to red chromogenesis. Agar slant : Moist heavy growth. Broth : Turbid with no pellicle. Litmus milk : No change to slightly alkaline. Potato : Abundant growth, usually tan, yellow or pink. Indole : Not produced. Hydrogen sulfide : Produced. Acid : Not produced from carbohydrate. Nitrite : Produced.

(3) Gram-Positive Strain Producing Spores. S42K2.

According to "Manual of Microbiological Methods", if bacteria can resist heating to 85°C for 10 minutes, endospores in such bacterial cells may be regarded as probably present. According to "Bergy's Manual", the rod-shaped bacteria possessing endospores belong to Family Bacillaceae in Order Eubacteriales. There are two genera Bacillus and Clostridium in this Family. The species of Bacillus are aerobic or facultatively anaerobic and catalase positive while those of Clostridium are anaerobic or aerotolerant and catalase-negative. Our strain S42K2 was aerobic, rod-shaped and catalase-positive, in addition, being able to resist heating 85°C for 10 minutes. Thus, S42K2 was considered to belong to Genus Bacillus. But the sporangia and endospores in this bacteria were not observed with a electron-microscope and a spore-staining method. The strain S42K2 did not grow 7 % NaCl broth. As there were not found the species in this Genus related to our strain S42K2, the strain was assigned to viriant of Bacillus.

Thirteen strains of bacteria obtained from oil-soaked soils were found to belong to the species of Genera Pseudomonas, Alcaligenes, Achromobacter, Corynebacterium and Bacillus. These bacteria were able to assimilate various substances. All of them assimilated n-alkanes but not aromatics. It was likely that Gram-negative strains belonging to Pseudomonas, Alcaligenes and Achromobacter prefered n-alkanes with less carbon numbers than n-tridecane (C_{13}) and Gram-positive ones belonging to Corynebacterium and Bacillus did those with carbon numbers more than n-hexadecane (C_{16}). No preference in assimilation was obserbed between even and odd

number of carbon in n-alkanes. Interestingly, all bacteria except S30K1 refused assimilation of n-tetradecane (C_{14}).

Most of the strains were able to utilize ethanol, while all strains were not able to do methanol. Gram-negative strains assimilated diols and Gram-positive ones did ethanol. Similar facts have been found by many investigators. T. Harada et al. reported production of succinoglucan from 1, 2-ethanediol by Alcaligenes faecalis var. myxogenes[21] and the utilization of ethanol by Corynebacterium sp. E17[22].

Most of our strains were capable of assimilating palmitic acid (the higher fatty acid), but not caproic acid (the lower fatty acid) except S34K1. It is of interest in our strains that in spite of the incapability in assimilating monobasic caproic acid with six carbon atoms, the corresponding dibasic acid, adipic acid, was utilized widely. Acetate was utilized by S40K1, S42K1 and S43K1. Corynebacterium acetophilm A51[23] which produced glutamate from acetate were found by T. Harada.

The utilization of polyvinylalcohol ($\overline{P}=500$) and a commercial neutral detergent by our strains were unsuccessfully examined in expectation of the cleavage of these substances.

H. Iizuka et al. found the bacteria (Pseudomonas, Alcaligenes, Flavobacterium, Brevibacterium and Corynebacterium) which utilized kerosene and crude oils as a sole carbon source and some species in these genera to produce glutamic acid. I. Shiio et al [24]. found that Corynebacterium hydroclastus and Corynebacterium oleophilus yielded 5.1 to 281.4 μg/ml of glutamate. T. Iguchi[25] found that Corynebacterium petrophilus yielded 13.0 mg/ml of glutamate in a 3.9% (w/v) n-hexadecane-including medium.

Our bacteria have not been examined at all for such a product as glutamate. But there could be bacteria capable of producing valuabe products from the carbon source used in tlhe present paper.

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