Studies on the Utilization of Hydrocarbons by Microorganisms

Part II. Taxonomical Studies on the Amino Acids-producing Bacteria from Hydrocarbons*

By Koichi YAMADA, Joji TAKAHASHI and Kaetu KOBAYASHI

Department of Agricultural Chemistry, Faculty of Agriculture, The
University of Tokyo, Tokyo

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Taxonomical studies on ten strains of hydrocarbon-utilizing bacteria reported in previous paper, which produced various kinds of amino acid, were carried out. They were *Achromobacter cycloclastes*, *Achromobacter delmarvae*, *Bacillus* species, *Corynebacterium* species, *Micrococcus* species. Many of them were not identical with the species which are described in Bergey's Manual of 7th Edition.

In previous paper¹⁾ were reported on the production of amino acids from hydrocarbons by bacteria, which were isolated from many kinds of soil in Japan.

In that report we described the amino acids-producing eleven strains with the sign of S7B1, S10B1, S10B2, S11B1, S12B2, S17B1, S20B1, S31B1, S39B1, S48B3 and S64B1.

In this paper, the taxonomical studies of these strains shall be reported except No. S7B1, because this strain is remarkable for its growth in the medium of hydrocarbons and will be reported in next paper²⁾ including its taxonomical study.

EXPERIMENTAL

Diagnostic tests were carried out according to the "Manual of Microbiological Methods", 1957, Society of American Bacteriologists, and referred to "Bergey's

Manual of Determinative Bacteriology", 7th Ed., 1957, and partly to the methods of other recent studies^{3,4)}.

(1) Cell Morphology.

Diagnostic tests for morphology, cell size, motility and Gram stain were carried out with the cells cultivated for 12 to 18 hrs. at 30°C on slanted nutrient agar. After 7 to 10 days' cultivation, irregular shape and Gram stain were repeatedly tested. Flagella were observed by the staining method of Nishizawa and Sugahara⁵⁾ and the electron-microscopic technique.

(2) Cultural Characteristics.

Characteristics were observed on plated nutrient agar after 7 to 10 days' cultivation and on slanted nutrient agar after 3 to 5 days. Temperature used for incubation of bacteria was 30°C except in the case of gelatin liquefaction tests.

(3) Physiological Characteristics.

A) Reduction of nitrates: Nutrient broth containing 0.1% KNO₃ and medium of succinic-nitrates were used for this test. After 1, 3 and 5 days' cultivation, nitrite was detected with α -naphthylamine and sul-

* This work was presented at the Meeting of Kanto Division of the Agricultural Chemical Society of Japan, March 2, 1963.

1) J. Takahashi, K. Kobayashi, Y. Imada and K. Yamada, This Journal, **28**, 390 (1963).

2) J. Takahashi, K. Kobayashi, Y. Kawabata and K. Yamada, "Production of Bacterial Cell from Hydrocarbons", the Meeting of the Agricultural Chemical Society of Japan, Tokyo, April 4, 1963.

3) H. Iizuka and K. Komagata, *J. Gen. Appl. Microbiol.*, **9**, 73 (1963).

4) H. Iizuka and K. Komagata, *ibid.*, **9**, 83 (1963).

5) Denken-Gakuyu Kai. "Saikingaku Zitsushu Teiyo" (in Japanese), Maruzen (1955).

fanilic acid.

B) Production of indole: After 1, 3 and 5 days' cultivation in 1% peptone water, indole was detected by Kovac's reagent.

C) Production of hydrogen sulfide: For detection of hydrogen sulfide we used the method with the filter paper soaked in lead acetate for the gases out of nutrient broth containing 0.05% cystine.

D) Hydrolysis of starch: After 2 and 4 day's cultivation on plated nutrient agar containing 0.2% starch, hydrolysis of starch was detected by the method of iodine reaction.

E) Catalase test: The cells, which were cultivated for 18 to 24 hrs on slanted nutrient agar, were added to 3% H_2O_2 solution. If foaming occurred, we found the test positive.

F) Voges-Proskauer test (V. P. test): V. P. test was carried out according to the conventional method.

G) Relation to pH: Growth at pH of 3, 4, 5, 6, 7, 8 and 9 was compared using nutrient broth as a culture medium.

H) Optimum temperature for growth: Growth at temperatures of 20°, 25°, 30°, 37° and 42°C was compared using nutrient broth.

(4) Cleavage of Sugar.

A) Cleavage of sugar is tested by liquid culture method: The bacteria were inoculated into media (peptone 0.3%, NaCl 0.25%, sugar 1%, pH 7.2 added with B.C.P. as indicator) in Durham tubes, incubated at 30°C for 2, 4 and 7 days, and then observed whether acid or gas was produced from the sugar.

B) Aerobical and anaerobical cleavage of sugar: According to Hugh & Leifson's method⁶⁾ tests were carried out with stab culture in semi-fluid media of low agar concentration, with or without liquid paraffin. Aerobical cleavage of sugar was more evident than fermentation test.

C) Assimilation of carbon compounds: After the cultivation at 30°C for 7 days, assimilation of carbon compounds was tested by measuring turbidity of cultivated broth. The media used were composed of NH_4NO_3 1.0 g, K_2HPO_4 1.0 g, $MgSO_4$ 0.5 g, 1,000 ml distilled water and the carbon compound to be tested. Concentration of carbon compounds added was 1% in case of carbohydrates and salts of organic acids, and 0.2% in case of aromatic acids.

D) Utilization of hydrocarbons: After the cultiva-

tion at 30°C, for 6 days, utilization of hydrocarbons was observed by measuring the packed volumes of wet cells centrifuged down at $5,200\times G$ for 15 minutes.

RESULTS AND DISCUSSIONS

Eleven strains of bacteria isolated from soil, S7B1, S10B1, S10B2, S11B1, S12B2, S17B1, S20B1, S31B1, S39B1, B48B3, and S64B1, which had produced comparatively large amounts of amino acids from hydrocarbon, were reported in previous paper (see, Table I).

1) Diagnostic Studies of Strains S10B1, S10B2, S11B1, S12B2.

Results of diagnostic tests for strains, S10B1, S10B2, S11B1, S12B2, are shown in Table II, following the style of the description of Bergey's Manual, 7th Ed.

Our strains are curved, knobbed, or branched, non-motile, Gram-positive rods and incapable of forming endospores. Therefore, it is confirmed that they belong to Family *Corynebacteriaceae*.

In Family *Corynebacteriaceae*, there are six genera, i.e., *Corynebacterium*, *Listeria*, *Erysipelothrix*, *Microbacterium*, *Cellulomonas*, and *Arthrobacter*. *Listeria* contains one strain, *Listeria monocytogenes*. According to the description of Bergey's Manual, it is motile by means of peritrichous flagella. Consequently, our strains cannot be included in *Listeria*, because they have no flagellum. As our strains do not form filaments, and are incapable of producing acid from lactose and of decomposing cellulose, they cannot be included in *Erysipelothrix*, *Microbacterium*, or *Cellulomonas*. They probably belong to *Corynebacterium* or *Arthrobacter*, but species of *Arthrobacter* are able to liquefy gelatin except *Arthrobacter terregens*, which is not able to grow on ordinary media. On the contrary, our strains are incapable of liquefying gelatin and capable of growing on ordinary media. Therefore, four strains above

6) R. Hugh and Leifson, *J. Bacteriol.*, **66**, 24 (1953).

TABLE I. RESULTS OF THE DETERMINATION OF AMINO ACIDS BY MICROBIOASSEY*¹

Strain No.	Medium used	Amounts of amino acids produced (mg/l)						
		alanine	aspartic acid	glutamic acid	glycine	isoleucine	lysine	valine
S7B1	U	17.0	23.0	23.0	—	15.0	—	38.0
S10B1	U	18.5	0	485.5	2.0	—	7.5	—
S10B2	U	25.0	0	200.0	8.5	—	—	—
S11B1	U	—	0	98.0	0.5	—	—	—
S12B2	M-2* ²	2.5	10.0	42.0	8.0	—	2.4	—
S17B1	U	—	0	54.0	4.0	—	23.9	—
S20B1	U	8.0	0* ³	0* ³	8.0	—	3.2	—
S31B1	M-2* ²	50.0	11.0	74.0	7.0	—	2.4	—
S39B1	U	1.5	0* ³	0* ³	0* ³	—	—	—
S48B3	U	38.0	10.5	94.5	8.5	—	17.0	—
S64B1	U	42.0	10.5	24.5	1.5	—	—	—

*¹: *Lactobacillus arabinosus* and *Leuconostoc mesenteroides* were used.

*²: pH of broth was controlled to 7.0 by adding aqueous ammonia during the cultivation.

*³: Microbioassay was hindered by some unknown factor in the culture broth.

M-2 medium: kerosene 3.5%, liquid paraffin 3.5%, NH_4NO_3 0.5%, K_2HPO_4 0.25%, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.1%, Tween-20, 0.05%.

U medium: 0.3% urea was used added to M-2 medium instead of 0.5% NH_4NO_3 .

mentioned belong possibly to Genus *Corynebacterium* described in Bergey's Manual.

As our strains are non-motile, aerobic or facultatively anaerobic, incapable of forming acid in the fermentation of sugar, capable of producing nitrite from nitrate and capable of liquefying gelatin, so only *Corynebacterium pseudodiphtheriticum*, *Corynebacterium nephridii*, and *Corynebacterium equi* can be considered as species related with our strains. However, these species are found to be different from our strains in respect that these species produce indole.

From the above experimental facts, four of our strains are determined to belong to Genus *Corynebacterium*, however there are no species (in Bergey's Manual, 7th Ed.) that confirm to our strains, S10B1, S10B2, S11B1 and S12B2. It is the fact that S10B1 is similar to S12B2 and that S10B2 is similar to S11B1. The strains, S10B1 and S12B2, may be similar to *Corynebacterium hydrocarbonclastus* Iizuka et Komagata⁷⁾.

2) Diagnostic Studies of Strains S17B1, S20B1, S31B1, S48B3.

Results of studies are shown in Table III and IV, together with the comparable description in Bergey's Manual, 7th Ed.

Our strains, S17B1, S20B1, S31B1 and S48B3, are Gram-negative rods, and the first one is non-motile and the other three strains are motile by means of peritrichous flagella. All are capable of growing on ordinary peptone media, incapable of ferment glucose anaerobically, and have aerobic properties. Therefore, these four strains belong possibly to Family *Achromobacteraceae* described in Bergey's Manual.

In Family *Achromobacteraceae*, there are five genera, i.e., *Alcaligenes*, *Achromobacter*, *Flavobacterium*, *Agarobacterium*, and *Be-neckeia*. Our strains do not attack agar but unusually show chromogenesis on peptone media, therefore it is possible that our strains belong to Genus *Alcaligenes* or *Achromobacter*. If our strains change milk alkaline and do not produce acid from sugar, then they probably belong to Genus *Alcaligenes*. But one of our strains, S17B1,

7) H. Iizuka and K. Komagata, The 188th Monthly Meeting of the Kanto Division of the Agricultural Chemical Society of Japan, November 22 (1958).

Assimilation of carbon compounds	glucose	no growth	no growth	no growth	no growth	no growth
	gluconate	growth	no growth	no growth	no growth	no growth
Kinds of hydrocarbons utilized for growth	2-keto gluconate	no growth	no growth	no growth	no growth	no growth
	citrate	growth	growth	growth	growth	growth
	succinate	growth	growth	growth	growth	growth
	benzoate	growth	no growth	no growth	no growth	no growth
	salicylate	no growth	no growth	no growth	no growth	no growth
	<i>m</i> -OH-benzoate	no growth	no growth	no growth	no growth	no growth
	<i>p</i> -OH-benzoate	no growth	no growth	no growth	no growth	no growth
Kinds of hydrocarbons scantily utilized or not utilized for growth	protocatechuate	growth	no growth	no growth	no growth	no growth
	gentisate	no growth	no growth	no growth	no growth	no growth
Kinds of hydrocarbons scantily utilized or not utilized for growth	anthranilate	no growth	no growth	no growth	no growth	no growth
Growth on kerosene media		<i>n</i> -undecane, <i>n</i> -dodecane, <i>n</i> -tridecane, <i>n</i> -tetradecane, <i>n</i> -hexadecane, <i>n</i> -eicosane, <i>n</i> -docosane, kerosene	<i>n</i> -decane, <i>n</i> -undecane, <i>n</i> -dodecane, <i>n</i> -tridecane, <i>n</i> -tetradecane, <i>n</i> -hexadecane, <i>n</i> -octadecane, <i>n</i> -eicosane, <i>n</i> -docosane, dodecene-1, tetradecene-1, hexadecene-1, octadecene-1, tricosene-11, kerosene, light oil	<i>n</i> -pentane, <i>n</i> -hexane, <i>n</i> -heptane, <i>n</i> -octane, <i>n</i> -nonane, <i>n</i> -decane, <i>n</i> -undecane, <i>n</i> -dodecane, <i>n</i> -tridecane, <i>n</i> -tetradecane, <i>n</i> -hexadecane, <i>n</i> -octadecane, <i>n</i> -eicosane, <i>n</i> -docosane, dodecene-1, tetradecene-1, hexadecene-1, octadecene-1, tricosene-11, kerosene, light oil	<i>n</i> -pentane, <i>n</i> -hexane, <i>n</i> -heptane, <i>n</i> -octane, <i>n</i> -nonane, <i>n</i> -decane, <i>n</i> -undecane, <i>n</i> -dodecane, <i>n</i> -tridecane, <i>n</i> -tetradecane, <i>n</i> -hexadecane, <i>n</i> -octadecane, <i>n</i> -eicosane, <i>n</i> -docosane, dodecene-1, tetradecene-1, hexadecene-1, octadecene-1, tricosene-11, kerosene, light oil	<i>n</i> -pentane, <i>n</i> -hexane, <i>n</i> -heptane, <i>n</i> -octane, <i>n</i> -nonane, <i>n</i> -decane, <i>n</i> -undecane, <i>n</i> -dodecane, <i>n</i> -tridecane, <i>n</i> -tetradecane, <i>n</i> -hexadecane, <i>n</i> -octadecane, <i>n</i> -eicosane, <i>n</i> -docosane, dodecene-1, tetradecene-1, hexadecene-1, octadecene-1, tricosene-11, kerosene, light oil
		<i>n</i> -pentane, <i>n</i> -hexane, <i>n</i> -heptane, <i>n</i> -octane, <i>n</i> -nonane, <i>n</i> -decane, isopentane, 2-methylpentane, 3-methylpentane, pentane-1, pentene-2, hexene-1, hexene-2, heptene-1, heptene-2, octene-1, octene-2, decene-1, dodecene-1, tetradecene-1, hexadecene-1, ethylcyclohexane, cyclohexane, liquid paraffin	<i>n</i> -pentane, <i>n</i> -hexane, <i>n</i> -heptane, <i>n</i> -octane, <i>n</i> -nonane, <i>n</i> -decane, isopentane, 2-methylpentane, 3-methylpentane, pentane-1, pentene-2, hexene-1, hexene-2, heptene-1, heptene-2, octene-1, octene-2, decene-1, dodecene-1, tetradecene-1, hexadecene-1, ethylcyclohexane, cyclohexane, liquid paraffin	<i>n</i> -pentane, <i>n</i> -hexane, <i>n</i> -heptane, <i>n</i> -octane, <i>n</i> -nonane, <i>n</i> -decane, isopentane, 2-methylpentane, 3-methylpentane, pentane-1, pentene-2, hexene-1, hexene-2, heptene-1, heptene-2, octene-1, octene-2, decene-1, dodecene-1, tetradecene-1, hexadecene-1, ethylcyclohexane, cyclohexane, liquid paraffin	<i>n</i> -pentane, <i>n</i> -hexane, <i>n</i> -heptane, <i>n</i> -octane, <i>n</i> -nonane, <i>n</i> -decane, isopentane, 2-methylpentane, 3-methylpentane, pentane-1, pentene-2, hexene-1, hexene-2, heptene-1, heptene-2, octene-1, octene-2, decene-1, dodecene-1, tetradecene-1, hexadecene-1, ethylcyclohexane, cyclohexane, liquid paraffin	<i>n</i> -pentane, <i>n</i> -hexane, <i>n</i> -heptane, <i>n</i> -octane, <i>n</i> -nonane, <i>n</i> -decane, isopentane, 2-methylpentane, 3-methylpentane, pentane-1, pentene-2, hexene-1, hexene-2, heptene-1, heptene-2, octene-1, octene-2, decene-1, dodecene-1, tetradecene-1, hexadecene-1, ethylcyclohexane, cyclohexane, liquid paraffin
Growth on kerosene media		abundant growth	abundant growth	abundant growth	abundant growth	abundant growth
	Relation to pH	7.0~9.0, no growth at pH 5.0	7.0~9.0, no growth at pH 5.0	6.0~9.0, no growth at pH 5.0	6.0~9.0, no growth at pH 5.0	6.0~9.0, no growth at pH 5.0
Growth on kerosene media		abundant growth	abundant growth	abundant growth	abundant growth	abundant growth
	Relation to free oxygen	aerobic	aerobic	aerobic	aerobic	aerobic
Growth on kerosene media		25~30°C, scant growth at 37°C	25~30°C, scant growth at 37°C	25~30°C, scant growth at 37°C	25~30°C, scant growth at 37°C	25~30°C, scant growth at 37°C
	Optimum temperature	positive	positive	positive	positive	positive
Growth on kerosene media		isolated from garden solid, Saitama	isolated from garden solid, Saitama	isolated from garden solid, Saitama	isolated from garden solid, Saitama	isolated from garden solid, Saitama
	Catalase	positive	positive	positive	positive	positive
Growth on kerosene media		isolated from garden solid, Saitama	isolated from garden solid, Saitama	isolated from garden solid, Saitama	isolated from garden solid, Saitama	isolated from garden solid, Saitama
	Source	positive	positive	positive	positive	positive

TABLE III. DESCRIPTIVE CHART OF STRAIN No. S17B1

	<i>Achromobacter delmarvae</i>	S 17 B 1
Morphological characteristics	rods $0.75 \mu \times 1.5 \mu$ non-motile absent negative gram-stain	rods $0.6 \mu \times 1.4 \mu$ non-motile absent negative
Agar -colonies	circular smooth entire raised bluish white glistening translucent	circular smooth entire convex pale yellowish brown glistening opalescent
Agar slant	growth form lustre color odor consistency medium	moderate filiform glistening pale yellowish brown not tested butyrous unchanged
Nutrient broth	surface growth clouding sediment	ring or fragile pellicle strongly turbid not tested
Gelatin stab	growth liquefaction	best growth at top none
Action on milk	coagulation, acid	unchanged, acid
Action on nitrate	nitrite	nitrite but no gas
Hydrogen sulfid	negative	positive
Indole production	negative	negative
V. P. test	not described	negative
Hydrolysis of starch	not described	not tested
Cleavage of sugar in liquid culture	glycerol xylose glucose lactose sucrose starch	no acid, no gas no acid, no gas no acid, no gas no acid, no gas no acid, no gas no acid, no gas no acid, no gas
Cleavage of sugar according to Hugh & Leifson's method	aerobic { glucose { lactose an- { glucose { lactose	not described not described not described not described acid but no gas acid but no gas acid but no gas no acid, no gas no acid, no gas
Aissimilation of carbon compounds	glucose gluconate 2-ketogluconate citrate succinate benzoate salicylate m-OH-benzoate p-OH-benzoate protocatechuate gentisate anthranilate	growth growth growth growth growth growth no growth no growth no growth growth growth growth
Growth on kerosene media	not described	abundant growth
Relation to pH	7.0	6.0~9.0 no growth at pH 5.0
Relation to free oxygen	facultatively anaerobic	aerobic
Optimum temperature	26~30°C, very slight growth at 37°C	30°C, scanty growth at 37°C
Catalase	not described	positive
Source	isolated from fresh strawberries	isolated from farm soil, Tokyo

TABLE IV. DESCRIPTIVE CHART OF STRAIN No. S20B1, S31B1, S48B3

		<i>Achromobacter cycloclastes</i>		
		S 20 B 1	S 31 B 1	S 48 B 3
Morphological characteristics	{ form size motility flagella }	rods 1.0~1.5 μ × 1.5~8.0 μ motile peritrichous negative	rods 0.4 μ × 0.8~1.2 μ motile peritrichous negative	rods 0.4~0.6 μ × 0.8~1.0 μ motile peritrichous negative
	{ gram-stain }	negative	negative	negative
Agar colonies	{ form surface edge }	circular to amoeboid smooth entire	circular smooth entire	circular smooth entire
	{ elevation color lustre }	flat to convex white glistening translucent with opaque center	raised to convex pale yellowish brown glistening opalescent	raised pale yellowish brown glistening opalescent
Agar slant	{ growth form lustre }	moderate filiform, undulate glistening pale buff	moderate filiform glistening pale yellowish gray	moderate filiform glistening pale yellowish brown
	{ color odor consistency medium }	not described not described unchanged	not tested butyrous unchanged	not tested butyrous unchanged
Nutrient broth	{ surface growth clouding sediment }	none turbid none	fragile pellicle turbid none	none moderately turbid flocculent
	{ growth liquefaction }	best growth at top none unchanged, alkaline	best growth at top none unchanged, alkaline	best growth at top none unchanged, alkaline
Gelatin stab		nitrite or gas	nitrite or gas	nitrite or gas
Action on milk		positive	weakly positive	positive
Action on nitrate		negative	negative	negative
Hydrogen sulfide		negative	negative	negative
Indole production		negative	negative	negative
V. P. test		negative	negative	negative
Hydrolysis of starch	{ glycerol xylose }	no acid, no gas no acid, no gas	no acid, no gas no acid, no gas	no acid, no gas no acid, no gas
	{ glucose lactose sucrose }	no acid, no gas no acid, no gas no acid, no gas	no acid, no gas no acid, no gas no acid, no gas	no acid, no gas no acid, no gas no acid, no gas
Cleavage of sugar in liquid culture	{ starch }	not described	not described	not described
	{ glucose lactose }	not described	not described	not described
Cleavage of sugar according to Hugh & Liefson's method	{ aerobic }	not described	not described	not described
	{ anaerobic }	not described	not described	not described

(Continued)	<i>Achromobacter cycloclastes</i>			
		S 20B1	S 31B1	S 48B3
Assimilation of carbon compounds	glucose	no growth	no growth	no growth
	gluconate	no growth	no growth	no growth
	2-keto gluconate	growth	growth	no growth
	citrate	scanty or no growth	growth	no growth
	succinate	scanty or no growth	growth	no growth
	benzoate	no growth	no growth	no growth
	salicylate	no growth	no growth	no growth
	m-OH-benzoate	no growth	no growth	no growth
	p-OH-benzoate	no growth	no growth	no growth
	protocatechuate	no growth	no growth	no growth
Growth on kerosene media	gentisate	no growth	growth	no growth
	anthranilate	no growth	no growth	no growth
Relation to pH	not described	abundant growth	abundant growth	abundant growth
Relation to free oxygen	not described	6.0~9.0, no growth at pH 5.0	5.0~9.0, no growth at pH 4.0	5.0~9.0, no growth at pH 4.0
Optimum temperature	aerobic, facultative	aerobic	aerobic	aerobic
Catalase	30~35°C	25~30°C, no growth at 37°C	30°C, scanty or no growth at 42°C	25~30°C, no growth at 37°C
Source	not described	positive	positive	positive
	isolated from soil	isolated from farm soil, Tokyo	isolated from crude oil-soaked sandy soil, Yokohama	isolated from crude oil-soaked clayey soil, Akita

obviously turns milk acid and produces acid from glucose and lactose, so it belongs to Genus *Achromobacter* described in Bergey's Manual, 7th Ed: Besides, the latter three strains, S20B1, S31B1, S48B3, turn milk alkaline, and produce no acid from sugar, therefore, they may belong either to Genus *Alcaligenes* or *Achromobacter*. If these three strains belong to Genus *Alcaligenes*, *Alcaligenes faecalis* and *Alcaligenes recti* would be comparatively related with our strains in respect of their motile characters. But, our strains are different from these two species in other characters. Then our strains, S17B1, S20B1, S31B1, and S48B3, are concluded to belong to Genus *Achromobacter*.

The strain S17B1 is non-motile rods, and can liquefy gelatin and produce acid from glucose, therefore, only *Achromobacter eurydice* and *Achromobacter delmarvae* can be considered as species related with our strain. However, *Achromobacter eurydice* is different from S17B1 in the action on milk and nitrates. In spite of the fact above mentioned that *Achromobacter delmarvae* is the most similar species to S17B1 in Bergey's Manual, both are different in optical characteristics of agar colonies, activity of producing hydrogen sulfide, and reaction to pH indicator. There are no species which exactly confirm to S17B1 in Bergey's Manual, however, it is reasonable to say that S17B1 resembles *Achromobacter delmarvae*.

The latter three strains, S20B1, S31B1, and S48B3, are closely related to *Achromobacter cycloclastes* or *Achromobacter pestifer* in their characters of motility, non-liquefaction of gelatin, non-action on milk, and fermentation of sugar without producing any acid. However, our three strains attack phenol and naphthalene, so they may be identified as *Achromobacter cycloclastes* described in Bergey's Manual.

TABLE V. DESCRIPTIVE CHART OF STRAIN No. S39B1, S64B1

	<i>Bacillus lentus</i>	S 3 9 B 1	<i>Micrococcus calpogenes</i>	S 6 4 B 1
Morphological characteristics	form size motility flagella endospore, sporangia form of endospore gram-stain	rods $0.6\sim0.9\mu\times1.5\sim4.0\mu$ motile not described present ellipsoidal positive	cocci, in clumps or pairs not described not-motile absent absent absent positive	coccus $0.4\sim0.6\mu$ non-motile absent absent positive
Agar colonies	form surface edge elevation color lustre optical	small smooth round flat whitish not described translucent to opaque	circular smooth entire raised yellow glistening not described	circular rough, contoured ridged not tested raised yellow glistening opalescent
Agar slant	growth form lustre color consistency medium	moderate filiform glistening gray white butyrous unchanged	not described filiform glistening yellow streak not described not described	scanty filiform glistening yellow not tested not tested
Nutrient broth	surface growth clouding sediment	scanty almost clear none	not described moderately turbid slight, granular sediment	ring almost clear none
Gelatin stab	liquefaction	none	none	none
Action on milk		coagulated, acid	unchanged	coagulated, neutral
Action on nitrate		neither nitrite nor gas	nitrite	nitrite but no gas
Hydrogen sulfide		negative	negative	negative
Indole production		negative	negative	negative
V. P. test		positive	not described	negative
Hydrolysis of starch		positive	negative	positive
Cleavage of sugar in liquid culture	glycerol xylose glucose lactose sucrose starch	no acid, no gas acid but no gas acid but no gas no acid, no gas acid but no gas acid but no gas	no acid, no gas no acid, no gas no acid, no gas no acid, no gas no acid, no gas no acid, no gas	no acid, no gas no acid, no gas no acid, no gas no acid, no gas no acid, no gas no acid, no gas
Cleavage of sugar according to Hingh & Leifson's method	glucose lactose glucose glucose lactose	acid but no gas not described not described not described not described	not described not described not described not described not described	no acid, no gas no acid, no gas no acid, no gas no acid, no gas no acid, no gas

	Assimilation of carbon compounds	Growth on kerosene media	Relation to pH	Relation to free oxygen	Optimum temperature	Catalase	Source		
								<i>Bacillus lentus</i>	<i>Micrococcus colpogenes</i>
	glucose							S 39 B 1	S 64 B 1
	gluconate							no growth	no growth
	2-keto gluconate							no growth	no growth
	citrate							growth	no growth
	succinate							no growth	no growth
	benzoate							no growth	no growth
	salicylate							no growth	no growth
	<i>m</i> -OH-benzoate							no growth	no growth
	<i>p</i> -OH-benzoate							no growth	no growth
	protocatechuate							no growth	no growth
	gentisate							no growth	no growth
	anthranilate							no growth	no growth
								moderate growth	moderate growth
								not tested	6.0~8.0, no growth at pH 5.0 and pH 9.0
								aerobic	aerobic
								25~30°C, no growth at 37°C	25~50°C, no growth at 37°C
								positive	positive
								isolated from crude oil-soaked wet soil, Akita	isolated from crude oil-soaked clayey soil, Akita
								no growth at 45°C	does not grow at 4°C
								not described	not described
								isolated from soil	isolated from marine mud from the Gulf of Mexico

3) Diagnostic Studies of Strain S39B1.

This strain S39B1 as shown in Table V, is motile by means of peritrichous flagella, unbranched straight rods, Gram-positive, and capable of forming endospores. Therefore, it belongs obviously to Family *Bacillaceae* described in Bergey's Manual. As our strain is aerobic and catalase-positive, so it belongs to Genus *Bacillus*.

Our strain forms ellipsoidal spores and its sporangia are not definitely swollen but diameter of its vegetative rods is less than 0.9 micron, and is capable of hydrolyzing starch, fermenting glucose, producing urease, and incapable of reducing nitrate to nitrite. The strain cannot be conformed to *Bacillus coagulans* in the character of good growth at 26°C. Therefore, only *Bacillus lentus* can be considered as species related with our strain. However, the species is found to be different from our strain in following characters: edge and color of agar colonies, color and consistency of agar slant cultures, clouding and sediment of nutrient broth, and action on milk. So it is possible that our strain is different from *Bacillus lentus*, but, among the species in Bergey's Manual, *Bacillus lentus* is the most similar species to our strain S39B1.

4) Diagnostic Studies of Strain S64B1.

This strain S64B1 shown in Table V is Gram-positive spheres and does not form endospores. Therefore, it belongs to Family *Micrococcaceae* described in Bergey's Manual.

Our strain is aerobic and does not shape in regular masses, and does not produce acid anaerobically from glucose. Consequently, our strain is confirmed to belong to Genus *Micrococcus*.

Our strain reduces nitrate to nitrite but not to gas, does not produce pink, orange-red, or red pigment on agar media, not liquefy gelatin and not ferment glucose to acid. So

only *Micrococcus colpogenes* can be considered as species related with S64B1. However, *Micrococcus colpogenes* is found to be different from S64B1 in the following characters: surface of agar colonies, nutrient broth, action on milk and hydrolysis of starch. Thus, our strain S64B1 can not be identified

with *Micrococcus colpogenes*, while it is the most similar species to S64B1.

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