Isolation and Identification of *n*-Butane-assimilating Bacterium[†]

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A bacterial strain capable of assimilating gaseous *n*-alkanes was newly isolated from activated sludge by enrichment culture technique using *n*-butane as the sole carbon source. The strain was identified as *Pseudomonas butanovora* sp. nov. It utilized *n*-alkanes of C₂~C₉, primary alcohols and carboxylic acids for growth, but did not utilize sugars and C₁ compounds. The cell yields on gaseous *n*-alkanes, such as ethane, propane and *n*-butane, were 80% or more. The maximum specific growth rate on *n*-butane was 0.22 hr⁻¹ at 30°C, pH 7.0. Dried cells of this new isolate grown on *n*-butane contained 73% pure protein.

Gaseous normal alkanes ranging from methane to *n*-butane are known to be more advantageous than liquid alkanes in several points. Gaseous alkanes are less expensive than liquid ones and occur abundantly in the world. They are pure and clean not only from the scientific point but also from the psychological point.

Among the gaseous alkanes, methane is the cheapest and of the most abundant occurrence. Many notable results have been reported on the production of biomass from methane in pilot plant tests as well as in basic studies.^{1~5)} Especially, very promising results have been obtained by Harrison *et al.*⁶⁾ by using a mixed culture of a *Methylobacter* sp. with a methanol utilizer. However, the production rates of biomass from gaseous substrates are limited by the transfer rates of those gases into culture fluids, and a fermenter having much higher rate of mass transfer than ever used is required to industrialize the biomass production from methane, since the transfer rate of methane is relatively low.

Gaseous normal alkanes such as ethane, propane and *n*-butane are more advantageous

than methane in terms of the transfer rate. The transfer rates of those alkanes into water are 1.5~2.0 times as high as that of methane under the same conditions.⁷⁾ That is, the load on a fermenter for supplying the same mass of gaseous substrates is reduced to 1/2~2/3 when gaseous alkanes other than methane are used as the growth substrate. In addition to this advantage, the yields of biomass theoretically expected on those alkanes are about 1.4 times as high as that expected on methane, provided that the yield of biomass is proportional to the amount of ATP obtained by the complete oxidation of each substrate.⁸⁾ Therefore, those gaseous alkanes are considered to be promising carbon sources for the production of biomass, if a few difficulties reported by forerunning workers can be overcome. These difficulties are to isolate a strain having much higher rate of growth and to achieve the accumulation of much higher concentration of cells.^{9~11)}

From the above points of view, a screening work has been carried out searching the strains capable of growing on ethane, propane and/or *n*-butane as the sole carbon source, and several strains having higher rates of growth than ever reported have been newly isolated.

In this paper, the procedures for the

[†] Production of Biomass from Gaseous *n*-Alkanes.
Part I.

isolation of those strains and biological properties of the most promising one of them are reported.

MATERIALS AND METHODS

Procedures for the isolation. Activated sludge and soil as the source of gaseous alkane-assimilating strains were sampled from oil refining plants. About 0.2 g of these samples were put into 20 ml of culture medium contained in 500 ml shaking flasks, then 40 ml of *n*-butane (or propane, ethane) and 30 ml of carbon dioxide were introduced into the flasks in place of the same volume of air. The shaking flask employed was stoppered with a rubber stopper, as reported in the previous paper,¹²⁾ and equipped with a side arm through which the cellular concentration of culture system was measured to check the growth rate. The culture medium employed was a mineral salts medium containing $(\text{NH}_4)_2\text{HPO}_4$ 8 g, $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ 2.5 g, KH_2PO_4 2 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5 g, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 30 mg, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 60 mg, $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ 60 μg , $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 15 μg and yeast extract 100 mg in 1000 ml of tap water, and the pH was adjusted to 7.1.

The culture systems thus prepared were incubated at 30°C with continuous shaking (125 oscills./min, 70 mm stroke) for 3–4 days, and 1-ml of the cultures, the optical densities of which exceeded 1- U.O.D., were transferred into 20 ml of fresh mineral salts medium contained in shaking flasks of the same kind as above. These culture systems were further incubated under the same cultural conditions as above, and the cultures, the maximum specific growth rates of which were in excess of 0.1 hr^{-1} , were selected for the succeeding enrichment cultures which were carried out by repeating the above procedures of cultivation for several times. The single colony isolation from the final one of the successive cultures was then carried out by using an ordinary plate culture technique in which mineral salts medium supplemented with agar 2% was employed. The plates were incubated in a closed chamber containing a 7:5:88 mixture of *n*-butane (or propane, ethane), carbon dioxide and air at 30°C.

Gaseous alkanes were purchased from Tokyo Kasei Kogyo Co. and more than 99% pure. The optical density was measured at 660 nm, and 1- U.O.D. was corresponding to the cellular concentration of 0.96 mg (dry basis)/ml.

Procedures for the identification. The cell form, cell size and gram-stain were examined on 12 and 24 hr old cells grown on nutrient agar (Difco) at 30°C, and the gram-stain was carried out by Hucker's modified method.¹³⁾ The motility was checked by a hanging drop method, and the flagellation was confirmed by Toda's staining method.¹⁴⁾ Poly- β -hydroxybutyrate was checked by staining smears of 12–72 hr old cells grown on Stanier medium¹⁵⁾ with Sudan Black. The pigmentation was tested by plate

cultures on *Pseudomonas* F (Difco) and *Pseudomonas* P (Difco) medium after 1–14 days incubation at 10°C, 15°C, 20°C, 30°C and 37°C, respectively.

Biochemical and physiological characteristics were examined according to the methods described by Stanier *et al.*,¹⁵⁾ and arginine dihydrolase was also checked by Møller's method.¹⁶⁾

DNA base composition was calculated from its thermal denaturation temperature (T_m) according to the procedure of Marmur and Doty,^{17,18)} and the T_m was measured by the method of Yamada and Komagata.¹⁹⁾

The diagnostic of the new isolate was carried out according to "Bergey's Manual of Determinative Bacteriology 8th Ed."²⁰⁾

Assimilation tests for various substrates. For testing the assimilation of such substrates as alcohols, liquid *n*-alkanes, organic acids and sugars, 50 mg of each substrate were added to 20 ml of mineral salts medium contained in 500 ml cotton stoppered shaking flasks before (in case of non-volatile substrates) or after (in case of volatile substrates) the sterilization. One loopful cells were then transferred into the culture medium from a 2 day old slant culture grown on *n*-butane, and incubated at 30°C with continuous shaking.

For testing gaseous *n*-alkanes and alkenes for the growth, on the other hand, a calculated volume of each substrate corresponding to 50 mg was introduced into 500 ml rubber stoppered shaking flasks containing 20 ml of mineral salts medium inoculated with one loopful cells.

The cells were harvested after the stationary phase of growth was attained, when a substrate was assimilated and the cellular growth was observed, and were dried *in vacuo* to determine the yield of cells. The yield factor, $Y_{x/s}$, was then calculated by dividing the amount of dried cells harvested not by the amount of substrate consumed but by the amount of substrate supplied (= 50 mg).

RESULTS AND DISCUSSION

Isolation of gaseous alkane-assimilating bacteria

Nine strains, the maximum specific growth rates of which were in excess of 0.1 hr^{-1} , were newly isolated. Seven of them were isolated from 56 soil samples, and the other two were isolated from 19 samples of activated sludge. The most potent strain of them was a *n*-butane-grown one, the maximum specific growth rate of which attained to 0.22 hr^{-1} at 30°C. This strain was tentatively designated as BuB-1211 and selected for further investigations.

FIG. 1. Photomicrographs of BuB-1211.

TABLE I. CHARACTERISTICS OF STRAIN BuB-1211

Morphological characteristics:
Rods, 0.6–0.8 × 1.1–2.4 μm, occurring singly.
Motile with monotrichous flagellum. Gram-negative.
Accumulation of poly-β-hydroxybutyrate: Accumulated.
Cultural characteristics:
Colonies on Nutrient Agar: Circular, smooth, entire, convex, glistening.
Nutrient Agar slant: Moderate growth, filiform, glistening.
Color of colonies on Pseudomonas F and Pseudomonas P medium: Pale yellow to brownish yellow, media unchanged.
Physiological characteristics:
Anaerobic growth: No growth.
Requirement of growth factor: Not required.
Maximum growth temperature: 42.5°C.
Optimum pH for growth: 5~8
Biochemical characteristics:
Catalase: Positive.
Oxidase: Positive.
Urease: Negative.
Arginine dihydrolase: Negative.
Oxidation of gluconate: Positive.
Decarboxylation of
Lysine: Negative.
Ornithine: Negative.
Arginine: Negative.
Nitrate reduction: Positive.
Denitrification: Positive.
Methyl red test: Negative.
Voges-Proskauer reaction: Negative.
Production of indole: Negative.
Production of hydrogen sulphide: Weakly positive.
Hydrolysis of starch: Negative.
Hydrolysis of gelatin: Negative.
Utilization of citrate:
Koser's medium: Positive.
Christensen's medium: Positive.
Assimilation of arginine and betaine: Not assimilated.

Carbon source for growth.

D-Xylose: Negative.
D-Glucose: Negative.
Geraniol: Negative.
L-Valine: Positive.
1,2-Ethanediol: Negative.
2,3-Butanediol: Positive.
Glycolate: Negative.
DL-Arginine: Negative.
GC-Content in DNA: 67.3%

Identification of Strain BuB-1211

Strain BuB-1211 was gram-negative rods as shown in Fig. 1-a. Size of cells were 0.6~0.8 × 1.1~2.4 μm. Cells grown on solid and liquid media were motile and their flagellation were polar monotrichous as shown in Fig. 1-b. This strain grew well aerobically on nutrient agar and chemically defined media, but did not anaerobically. Oxidase and catalase were positive. GC-Content in DNA was 67.3%.

Morphological, physiological and biochemical characteristics of BuB-1211 are summarized in Table I. These characteristics indicate that BuB-1211 belongs to the genus *Pseudomonas* and is capable of denitrification. Therefore, characteristics of the denitrifying species of *Pseudomonas* appeared in "Bergey's Manual of Determinative Bacteriology 8th Ed." are compared with those of BuB-1211 in Table II. None of the denitrifying species of *Pseudomonas* ever reported, as is clear from Table II, is identical with the new isolate. Accordingly, BuB-1211 is recognized as a new

TABLE II. CHARACTERISTICS OF DENITRIFYING *Pseudomonas* SPECIES AND BuB-1211

Characteristics	Strain										
	BuB-1211	<i>P. aeruginosa</i>	<i>P. fluorescens</i>	<i>P. chlororaphis</i>	<i>P. aureofaciens</i>	<i>P. stutzeri</i>	<i>P. mendocina</i>	<i>P. pseudomallei</i>	<i>P. caryophylli</i>	<i>P. mallei</i>	<i>P. solanacearum</i>
Accumulation of PHB ^a	+	-	-	-	-	-	-	+	+	+	+
Production of diffusible pigments	-	+	+	+	+	-	-	-	-	-	D
Growth at 41°C	+	+	-	-	-	+	+	+	+	+	-
Arginine dihydrolase	-	+	+	+	+	-	-	+	+	+	-
Hydrolysis of gelatin	-	+	+	+	+	-	-	+	-	+	-
Hydrolysis of starch	-	-	-	-	-	+	-	+	-	D ^b	-
Carbon sources for growth											
D-Glucose	-	+	+	+	+	+	+	+	+	+	+
D-Xylose	-	+	+	+	ND	-	-	-	+	+	-
DL-Arginine	-	+	+	+	+	-	+	+	+	+	-
1,2-Ethanediol	-	+	-	ND ^c	ND	+	+	-	-	ND	-
2,3-Butanediol	+	+	-	ND	ND	+	-	-	+	-	-
Glycolate	-	+	-	-	-	+	+	+	+	-	-
Geraniol	-	+	-	-	-	-	+	-	-	-	D
L-Valine	+	+	+	+	+	+	+	+	-	D	+
GC-Content in DNA (%)	67.3	67.3	59.4 ~61.3	63.5	63.6	60.7 ~66.3	62.8 ~64.3	69.5	65.3	69.0	66.5 ~68.0

^a Poly-β-hydroxybutyrate.
^b Reaction differs.
^c No description.

species of the genus *Pseudomonas* and is identified as *Pseudomonas butanovora* Takahashi and Yamada sp. nov. The type strain was deposited in the Inst. of Appl. Microbiol., Univ. of Tokyo, and the given strain number was IAM-12574.

Growth on various substrates

Among the organic compounds tested, as shown in Table III, *n*-alkanes ranging from C₂ to C₉, primary alcohols and carboxylic acids of C₂, C₃ and C₄, and polyvalent alcohols of C₃ and C₄ are utilized for the growth, while *n*-alkanes of C₁₀ and more, C₁ compounds, *n*-alkanes and sugars are not utilized. The yields of cells on gaseous *n*-alkanes such as ethane, propane and *n*-butane are rather high, and the values of $Y_{x/s}$ attain to 0.8 and more. It is notable that this new species can well utilize *n*-alkanes of C₅~C₉ which have been known to be hardly assimilated by microorganisms.²¹⁾

Composition of cells

Table IV shows the composition of dried

TABLE III. UTILIZATION OF VARIOUS SUBSTRATES BY *Pseudomonas butanovora* FOR GROWTH

Substrate	Yield factor ^a	Substrate	Yield factor
Methane	— ^b	Ethylene	—
Ethane	0.80	Propylene	—
Propane	0.82	1-Butene	—
<i>n</i> -Butane	0.83	2-Butene	—
<i>n</i> -Pentane	0.87	1,3-Butadiene	—
<i>n</i> -Hexane	0.73		
<i>n</i> -Heptane	0.49	1,2-Ethanediol	—
<i>n</i> -Octane	0.37	1,2-Propanediol	0.46
<i>n</i> -Nonane	0.04	2,3-Butanediol	0.43
<i>n</i> -Alkanes, C ₁₀ to C ₁₆	—	Glycerol	0.10
		D-Sorbitol	—
Methanol	—	Erythritol	—
Ethanol	0.57		
<i>n</i> -Propanol	0.95	L-Arabinose	—
<i>n</i> -Butanol	0.85	D-Xylose	—
		D-Glucose	—
Sodium acetate	0.16	D-Fructose	—
Sodium propionate	0.75	Sucrose	—
Sodium butyrate	0.80		
Sodium lactate	0.43		

^a gm-cell/gm-substrate.

^b Not utilized.

TABLE IV. COMPOSITION OF DRY CELLS OF *Pseudomonas butanovora*

Component	Content (%)
Crude protein ^a	77.69
(Pure protein)	72.81
Carbohydrates ^b	3.06
Crude fat	2.77
Crude fiber	0
Ash	11.07
Moisture	8.39

^a Determined by semimicro-Kjeldahl method.

^b Calculated from the amount of reducing sugars in acid hydrolyzate of the cells.

cells of *P. butanovora* grown on *n*-butane as the sole source of carbon and energy. It is notable that the content of protein in the cells is very high and that of carbohydrates is extremely low. The content of pure protein as high as 73% is one of the highest values ever obtained in the cells grown on gaseous alkanes, though slightly higher values have been reported in the cells produced from methanol.²²⁾ The elemental composition of the dried cells was C: 47.33%, H: 6.85% and N: 13.37%, and the content of nonprotein nitrogen calculated from the difference between total and protein nitrogen was 1.72%. This corresponds to the cellular nucleic acid content of 11.05%, assuming all nonprotein nitrogen is in nucleic acids.

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