Production of Fluorescent Compounds from Guanine by Microorganisms

By Isao TAKEDA and Shiro HAYAKAWA

Research Laboratory of Asahi Chemical Industry Co., Ltd.,
Tokyo

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One species of hydrocarbon utilizing bacteria was isolated from soil. This strain was named as *Achromobacter petrophilum* No. 4017. This bacterial species utilizes normal hydrocarbons with carbon chains of nC_{10} to nC_{18} , but does not utilize glucose or other carbohydrates. *Achromobacter petrophilum* forms small amounts of green-yellow, green-blue and violet fluorescent compounds in the medium containing *n*-hexadecane (nC_{16}) as a carbon source. The mutant strain, No. 4510, which requires hypoxanthine and thiamine for growth, was obtained from *Achromobacter petrophilum* No. 4017 by ultraviolet irradiation and formed considerable amounts of green-yellow fluorescent compound by the addition of guanine to the *n*-hexadecane medium. This fluorescent compound was crystallized from culture broth.

In the previous papers,^{1~8)} assimilation of hydrocarbons by yeast and bacteria has been reported by authors. The present paper will describe the accumulation of fluorescent compounds in the medium containing hydrocarbon by a mutant strain of *Achromobacter* isolated from soil. This strain requires hypoxanthine and thiamine for growth. Substitution of guanine or xanthine for hypoxanthine does not support growth. This mutant is a purine auxotroph and lacks 5'-guanylic acid reductase. The addition of guanine to the culture medium is necessary for the formation of the green-

yellow fluorescent compound.

EXPERIMENTAL

1) **Materials.** All hydrocarbons were purchased from Tokyo Kasei Chemicals with the exception of *n*-pentadecane obtained from Kanto Koatsu Chemicals. They were above 95% in purity and used without further purification. "Avicell" (Asahi Chemical Co., over 100 mesh) was used as cellulose powder for chromatography. DEAE-cellulose was purchased from Brown Co., Berlin.

2) **Microorganism.** Strain No. 4017 was isolated from soil on the following medium containing 1.5% agar (Wako Pharm. Co.). The medium was as follows; light oil or kerosene 30 ml, $(NH_4)_2SO_4$ 1.0 g, NH_4NO_3 1.0 g, KH_2PO_4 2.0 g, $NaHPO_4$ 2.0 g, $MgSO_4 \cdot 7H_2O$ 1.0 g, $FeSO_4 \cdot 7H_2O$ 0.005 g, $MnSO_4 \cdot 4H_2O$ 0.005 g, $ZnSO_4 \cdot 7H_2O$ 0.005 g, yeast extract (Difco) 1.0 g and H_2O 1,000 ml. pH was adjusted 7.0 with 1 N KOH.

3) **Mutation.** The minimal medium consisted of *n*-hexadecane 10 ml, KH_2PO_4 3.0 g, K_2HPO_4 7.0 g, $(NH_4)_2SO_4$ 1.0 g, $MgSO_4 \cdot 7H_2O$ 0.2 g, Na-citrate 0.5 g per 1 liter. Strain No. 4017 can grow in this minimal medium at 30°C. The cells were centrifuged and resuspended in the same medium. Ten ml of cell suspension (2.8×10^8 cells per ml) was placed in plate

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TABLE I IDENTIFICATION OF STRAIN No. 4017

Morphologically;	Rods, 0.7 to 0.9 by 0.8 to 1.6 μ , occurring in single and pairs. Normally not encapsulated, non-motile and gram negative.
Gelatin stab;	Crateriform liquefaction.
Agar colonies;	Circular, small, smooth, entire, glistening, slightly raised, whitish with no pigment.
Bouillon culture;	Moderate growth and the cells precipitate at the bottom. No characteristic odor.
Broth containing peptone;	Moderate growth, a pellicle is formed and cells precipitate at the bottom.
Potato-dextrose agar;	The colonies are circular, small, smooth entire glistening, slightly raised, whitish with no pigment.
Litmus milk;	Changed to alkaline after 5 days. There is no peptonization.
Indole;	Not produced.
Hydrogen sulfide;	Not produced.
Production of nitrite from nitrate;	Positive after 7 days.
Acids or gas produced from carbohydrate media;	No.
Ammonium produced from peptone;	Positive.
Ammonium chloride serves as a source of nitrogen, while urea is not utilized.	

and exposed to ultraviolet irradiation for 80 seconds (National Electric 15 W germicidal lamp) at a distance of 45 cm. After irradiation, the population density was approximately 2×10^5 viable cells per ml. The irradiated cells were resuspended in the minimal medium and cultured for 5 hr at 30°C. 500 unit per ml of penicillin G (Tōyō Jozo Co.) was added in accordance with Lederberg's method.⁹⁾ The survivors were aerobically incubated at 30°C in the medium composed of the minimal medium plus vitamin free casamino acids (Difco) 2.0 g, hypoxanthine 20 mg, uracil 20 mg, tryptophan 10 mg, cystine 10 mg, thiamine 500 μ g, riboflavin 500 μ g, para-aminobenzoic acid 20 μ g, folic acid 10 μ g, inositol 1,000 μ g and biotin 5 μ g per 1 liter H₂O. When the population reached 4×10^8 cells per ml, the cells were collected. The penicillin treatment was repeated four times by the method of Iguchi, Watanabe and Takeda.⁶⁾

4) **Analytical methods.** The green-yellow, green-blue and violet fluorescent compounds were determined as follow. The definite amounts of the culture filtrate were paperchromatographed. The bands corresponding to the green-yellow, green-blue and violet fluorescence were eluted with hot water and the eluates

were assayed spectrophotometrically at 400, 400 and 340 m μ respectively.

RESULTS AND DISCUSSION

Table I shows the properties of strain No. 4017. This strain was identified as *Achromobacter* species according to the Bergey's Manual of Determinative Bacteriology, 7th Edition, and named *Achromobacter petrophilum* No. 4017.

Achromobacter petrophilum was inoculated in the following medium; *n*-hexadecane 50 ml, KH₂PO₄ 1.0 g, MgSO₄·7H₂O 0.5 g, yeast extract (Difco) 10.0 g, (NH₄)₂SO₄ 4.0 g and CaCO₃ (precipitate) 2.0 g in 1 liter of water at pH 6.8 to 7.0. The bacteria were cultured in 50 ml of the sterilized medium at 30°C for 4 days in 500 ml shaking flasks. After 4 days incubation, small amounts of green-yellow, green-blue and violet fluorescent compounds were accumulated in culture broth. The paperchromatograms of the culture broth are shown in Fig. 1.

Achromobacter petrophilum No. 4017 was treat-

9) J. Lederberg and E. M. Lederberg, *J. Bacteriol.*, **63**, 399 (1952).

FIG. 1. Paperchromatograms of Fluorescent Compounds in Cultural Broth.

No. 1: Butanol-acetic acid-water (4:1:5)
 No. 2: Water saturated with benzylalcohol
 No. 3: 3% ammoniumchloride
 No. 4: Butanol-ethanol-water (10:3:7)
 G-Y; Green-yellow fluorescence
 G-B; Green-blue fluorescence
 V; Violet fluorescence

ed by ultraviolet irradiation and penicillin. The mutants obtained by the above mentioned treatments required a variety of growth factors including amino acids, purines and vitamins. Of these mutants, a strain that produced large amounts of the green-yellow and green-blue fluorescent compounds than the original strain in the culture medium described as above was

TABLE II. THE GROWTH OF *Achromobacter petrophilum* No. 4510 IN THE MINIMAL MEDIUM SUPPLEMENTED WITH PURINE BASES

Media	Growth*
Minimal medium ¹⁾	0
" plus Xanthine	0
" " Guanine	0
" " Hypoxanthine	+
" " Hypoxanthine, Xanthine	+
" " Hypoxanthine, Guanine	+
" " Adenine	+
Minimal medium ²⁾ plus Hypoxanthine	0

Minimal medium¹⁾ is as follows: *n*-hexadecane 5.0 ml, KH_2PO_4 3.0 g, K_2HPO_4 7.0 g, $(\text{NH}_4)_2\text{SO}_4$ 1.0, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.2 g, Na-citrate 0.5 g, casamino acid 2.0 g, tryptophan 10 mg, cystine 10 mg, thiamine 500 μg , para-aminobenzoic acid 20 μg , folic acid 10 μg , inositol 1,000 μg , riboflavin 500 μg and biotin 5 μg per 1 liter H_2O .

Minimal medium²⁾: Thiamine is omitted from minimal medium¹⁾. Purine bases were used at the concentration of 1 mg per 1 liter.

* + = growth, 0 = no growth

selected for further study. This strain, *Achromobacter petrophilum* No. 4510, is a purine auxotroph and lacks 5'-guanylic acid reductase, and requires thiamine for growth (Table II).

The mutant strain No. 4510 has the ability to produce considerable amounts of green-yellow fluorescent compound upon addition of guanine to a medium. Hypoxanthine addition also induces the formation of the fluorescent compound. The addition of xanthine to the culture broth was as effective as guanine for the formation of green-yellow fluorescent compound as shown in Table III. The

TABLE III. RELATIONSHIP BETWEEN THE ADDITION OF PURINE BASES AND THE FORMATION OF THE GREEN-YELLOW FLUORESCENT COMPOUND

Bases (500 mg/liter)	Green-yellow fluorescent compound	Growth*
None	0.01	##
Guanine-HCl	0.10	###
Xanthine	0.08	###
Hypoxanthine	0.04	###
Adenine	0.04	###
Guanosine	0.05	###

The values mentioned above show the reading at $-\log T$.

* ### = heavy growth.

The medium is composed of *n*-hexadecane 50.0 ml, KH_2PO_4 1.0 g, K_2HPO_4 1.0 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5 g, yeast extract 6.0 g, $(\text{NH}_4)_2\text{SO}_4$ 4.0 g, hypoxanthine 10 mg and sterilized CaCO_3 20.0 g per 1 liter at pH 6.8 to 7.0. The bacteria were cultured in 500 ml shaking flasks containing 50 ml medium at 30°C for 4 days.

TABLE IV. THE EFFECT OF THE GUANINE CONCENTRATION ON THE FORMATION OF THE GREEN-YELLOW FLUORESCENT COMPOUND

Guanine-HCl (mg/liter)	Green-yellow fluorescent compound
50	0.02
100	0.03
200	0.04
400	0.10
800	0.10

The culture medium and other conditions were the same as Table II.

concentration of guanine on the formation of this fluorescent compound was tested. Table IV shows that the addition of 400 mg of guanine per liter was the most effective. The relationship between the assimilation of hydrocarbons and the formation of the green-yellow fluorescent compound is shown in Table V. Normal

TABLE V. THE ASSIMILATION OF HYDROCARBONS AND THE FORMATION OF THE GREEN-YELLOW FLUORESCENT COMPOUND

Carbon source	Growth*	Fluorescent compound**
<i>n</i> -Decane	‡	
<i>n</i> -Decanoic acid	0	
<i>n</i> -Undecane	‡	
<i>n</i> -Dodecane	‡	+
Isododecane	+	
<i>n</i> -Tridecane	‡	+
<i>n</i> -Tetradecane	‡	+
Tetradecene-1	‡	+
<i>n</i> -Pentadecane	‡	+
Heptamethylnonane	±	
<i>n</i> -Hexadecane	‡	+
Hexadecene-1	‡	+
<i>n</i> -Octadecane	‡	+
Glucose	0	
Na-Succinate	0	
Glycerine	0	
Na-Acetate	‡	+

All hydrocarbons were used at a concentration of 50.0 ml per liter. The culture media and other conditions were the same as Table III, but *n*-hexadecane was omitted and guanine was added at a concentration of 500 mg per 1 liter.

* ‡=heavy growth, 0=no growth.

** +=formation of the fluorescent compound.

hydrocarbons from tridecane to octadecane showed good growth and good formation of the green-yellow fluorescent compound. Glucose, glycerine and Na-succinate were not assimilated by this mutant, but Na-acetate was utilized as a carbon source. The formation of the green-yellow fluorescent compound was stimulated by the addition of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and CaCO_3 into the culture broth as shown in Table VI and VII.

Achromobacter petrophilum No. 4510 was cul-

TABLE VI. THE CONCENTRATION OF $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ AND THE FORMATION OF THE GREEN-YELLOW FLUORESCENT COMPOUND

$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (g/liter)	Green-yellow fluorescent compound
0	0.01
0.5	0.02
1.0	0.06
5.0	0.10
10.0	0.08

The media were the same as Table III except for a concentration of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and an addition of guanine-HCl 500 mg/liter. The bacteria were cultured in 500 ml shaking flasks containing 50 ml medium at 30°C for 4 days.

TABLE VII. THE FORMATION OF THE GREEN-YELLOW FLUORESCENT COMPOUND AND CaCO_3

CaCO_3 (g/liter)	Green-yellow fluorescent compound
0	0
20	0.9

The media and the culture conditions were the same as Table III except for the additions of 400 mg of guanine and of 4.0 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ per liter.

tured at 30°C for 4 days in 50 ml of the sterilized medium described as follows in 500 ml shaking flasks; *n*-hexadecane 50 ml, KH_2PO_4 1.0 g, K_2HPO_4 1.0 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 4.0 g, yeast extract 6.0 g, $(\text{NH}_4)_2\text{SO}_4$ 4.0 g, Hypoxanthine 10 mg, guanine-HCl 500 mg, and sterilized CaCO_3 20 g per 1 liter H_2O at pH 6.8 to 7.0. Ammonium sulfate (60%) was added to 1 liter of the culture broth. The cells and the coagulated proteins were removed by filtration. Phenol (30 ml) was added to the filtrate (900 ml) and all the fluorescent compounds including the green-yellow, green-blue and violet fluorescent compounds were extracted according to the method of Crammer.¹⁰⁾ Ether (60 ml) and water (15 ml) were mixed perfectly with the phenol extracts and the aqueous layer was passed through a column of powdered cellulose (Avicell) equilibrated with water saturated with benzylalcohol. The fluorescent compounds

10) J. L. Crammer, *Nature*, **161**, 349 (1948).

were eluted from the column by water saturated with benzylalcohol. The aqueous layer, after removal of benzylalcohol by the addition of a small volume of ether, was adsorbed on a DEAE-cellulose column washed with 0.01 N HCl solution. The green-yellow fluorescent compound was eluted with 0.05 N HCl solution. The eluate was adsorbed to 300 mg of Norit A washed with 0.1 N HCl solution. Norit A was washed with acidic solution (pH 5) of HCl. The green-yellow fluorescent compound was eluted from Norit A with 60% ethanol solution containing 1% NH_4OH . The eluate was evaporated *in vacuo* to 5 ml and 0.5 ml of glacial acetic acid was added following the method of Forrest and McNutt.¹¹⁾ The precipitate was collected by centrifugation and discarded. The supernatant, on addition of methanol (5 ml), yielded pale yellow crystals. In this way, 3 mg of crystal were obtained from 1 liter of culture broth. The acicular crystals (Fig. 2) were reddish-yellow.

FIG. 2. Crystals of Green-yellow Fluorescent Compound.

The melting point of the crystal was found to be 291 to 295°C (decomp.). This compound showed no "folic acid" activity in the bioassay for *Streptococcus faecalis* R¹²⁾ or *Lactobacillus casei*.¹³⁾ The ultraviolet absorption spectra of this compound are shown in Fig. 3. The peaks

FIG. 3. Ultraviolet Spectrum of Green-yellow Fluorescent Compound.

FIG. 4. Infrared Spectrum of the Green-yellow Fluorescent Compound.

FIG. 5. The Ultraviolet Spectrum of the Green-blue Fluorescent Compound.

at 305 $\text{m}\mu$ in 0.1 N NaOH solution and at 310 $\text{m}\mu$ in 0.1 N HCl solution reveal a difference from the spectra obtained with pteridine derivatives. Fig. 4 shows the infrared absorption spectrum of the green-yellow fluorescent compound. As shown in Fig. 5, a green-blue fluorescent compound shows an ultraviolet spectrum similar to that of a green-yellow fluorescent compound.

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These green-yellow and green-blue fluorescent compounds are derived from guanine as shown in the preceding paragraphs. However, when 8-¹⁴C-guanine (10 μ Ci) was added to 10 ml of the culture medium in 24 hr after inoculation and incubated for 4 days, no label was incorporated into these green-yellow and green-blue fluorescent compounds. Thus, carbon 8 of guanine is not involved in the formation of these compounds. It has previously been reported that it is not involved in the formation of pteridine compounds from guanine.¹⁴⁻¹⁷⁾ The green-yellow fluorescent compound seems

to be different from pteridine derivatives produced by *Eremothecium ashbyii*,¹⁸⁾ *Mycobacterium smegmatis*,¹⁹⁾ and *Aspergillus oryzae*.²⁰⁾ The structure and physiological role of these compounds have yet to be elucidated.

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