MICROBIOLOGICAL STUDIES ON PETROLEUM AND NATURAL GAS

II. DETERMINATION OF PSEUDOMONADS ISOLATED FROM OIL-BRINES AND RELATED MATERIALS

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In the previous papers (1, 2), we briefly reported that about ten millions of aerobic bacteria and ten thousands of anaerobic bacteria in viable cell numbers were found in one ml of oil-brines arising from the oil-layers being located two hundred to seven hundred meters beneath, and the bacteria of the former group mainly belonged to genus *Pseudomonas*. Moreover we described that hydrocarbon-utilizing pseudomonads are widely distributed in petroleum zones in Japan (1, 2, 3). It is well-known that pseudomonads play an important role forming the main members of microflora of petroleum (4, 5, 6, 7). In this paper, we report the determinative studies on pseudomonads isolated from oil-brines and the other related materials. The ecological characteristics of pseudomonads in microflora of petroleum and natural gas will be described elsewhere.

MATERIALS AND METHODS

Isolation of microorganisms. Microorganisms were isolated from oil-brines and the other related materials obtained in Yabase, Higashiyama, and Nishiyama petroleum fields in Japan. Sampling of oil-brines was made at the oil-storage tanks, and direct sampling from oil-layers was carried out by using bottom-hole samplers which were employed in petroleum mining. The other materials such as flooding-water, crude oil, soil, etc. were obtained wherever we could collect them. The following culture media were used for the isolation of microorganisms. Nutrient agar consisted of per liter: peptone, 5.0 g; meat extract, 3.0 g; and agar, 20.0 g. NaCl-nutrient agar contained 3% NaCl in the nutrient agar. Glutamate agar consisted of per liter: glucose, 10.0 g; sodium glutamate, 5.0 g; K₂HPO₄, 1.0 g; MgSO₄·7H₂O, 0.2 g; KCl, 0.1 g; and agar, 20.0 g. *p*-Hydroxy-benzoate agar consisted of per liter: sodium *p*-hydroxy-benzoate, 2.0 g; NH₄NO₃, 1.0 g; K₂HPO₄, 1.0 g; MgSO₄·7H₂O, 0.5 g; and agar, 20.0 g. Kerosene agar consisted of per liter: kerosene, 10.0 g; NH₄Cl, 2.0 g; Na₂HPO₄, 1.0 g; KH₂PO₄, 0.5 g; MgSO₄·7H₂O, 0.5 g; NaCl, 2.0 g; and

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agar, 20.0g. Soil extract-glucose agar consisted of soil extract, 1000ml; glucose, 10.0g; K_2HPO_4 , 1.0g; and agar, 20.0g (soil extract was prepared by autoclaving 1kg of soil suspended in 1000ml of distilled water for 30min, followed by filtration, and filled to 1000ml by adding distilled water). Malt extract-yeast extract agar consisted of per liter: glucose, 4.0g; malt extract (Difco), 10.0g; yeast extract, 10.0g; and agar, 20.0g. All the media were adjusted to pH 7.2. Isolation of microorganisms was carried out by plating method at 30° for 7 to 10 days.

Differentiation and determination of microorganisms. The typical colonies appeared on each medium were picked up on the same agar slants, and then transferred to nutrient agar slant. Moreover, these isolates were differentiated and bacterial genera were assigned according to the method previously reported by the author (8). The typical pseudomonads were selected by such procedures. The determinative techniques employed were mainly those described in Manual of Microbiological Methods (9), and the detailed procedures were reported in other papers (10, 11). The pseudomonads selected were identified according to the original descriptions and Bergey's Manual of Determinative Bacteriology, 7th ed. (12).

RESULTS

Pseudomonads isolated from oil-brines and the other related materials were divided into two groups. In the first group, pseudomonads belonging to the achromogenic group, such as *Pseudomonas iners*, *Pseudomonas stutzeri*, and *Pseudomonas putrefaciens*, were included, and those of the fluorescent group, such as *Pseudomonas schuylkilliensis*, *Pseudomonas azotoformans*, *Pseudomonas nitroreducens*, and *Pseudomonas aeruginosa* were assigned to the second group. The new species of *Ps. iners* was identified by the present authors. The strains tested were all Gram-negative and motile. However, *Ps. azotoformans* No. 69 was non-motile. All the motile strains showed polar flagellation. Colonies on nutrient agar were mostly circular, entire and smooth. *Ps. stutzeri* had markedly wrinkled surface on glutamate agar plates. *Ps. putrefaciens* formed reddish brown colonies on nutrient agar plates. Pseudomonads belonging to the fluorescent group produced water-soluble greenish yellow fluorescent pigments. *Ps. aeruginosa*, *Ps. azotoformans*, *Ps. nitroreducens* and *Ps. stutzeri* grew anaerobically by performance of nitrate respiration. It was noteworthy that a large number of pseudomonads possessing the ability of nitrate respiration were found in oil-brines. Generally these pseudomonads produced minor amounts of acid from carbohydrates. *Ps. iners* failed to grow in absence of sodium chloride. The detailed taxonomical characteristics are described below.

DESCRIPTION

1. *Pseudomonas iners* nov. sp.

Strains: Nos. 52 and 92

Type strain: *Ps. iners* No. 52

The strains tested were Gram-negative, motile rods possessing polar flagellum. They required small amounts of sodium chloride for growth but did not grow in media containing over 9%. They failed to develop in synthetic media. The strains of this species readily oxidize steroids (13). This species belongs to the achromogenic group of genus *Pseudomonas* (10, 11).

Rods, 0.6 to 0.8 by 1.2 to 2.0 microns. Occurring singly, not in chains. Motile with a polar flagellum. Gram-negative.

Nutrient agar colonies: Circular, smooth, entire, raised, pale brown, glistening, butyrous.

Nutrient agar slant: Growth moderate, filiform, flat, glistening, very pale brown, translucent, medium unchanged.

Glutamate agar slant: No growth.

Nutrient broth: Ring, very slightly turbid.

Nutrient gelatin stab: No liquefaction.

Milk: Unchanged.

Litmus milk: Unchanged.

B. C. P. milk: Unchanged.

Nitrite not produced from nitrate in nitrate broth. No growth in succinate-nitrate broth.

Nitrate respiration: Negative.

Indole not produced.

Hydrogen sulfide may not be produced. (No growth in nutrient broth containing 0.05% cystein.).

Starch not hydrolyzed.

Acetylmethyl carbinol not produced.

No acid or gas from glycerol, xylose, glucose, sucrose, lactose and starch in peptone media. No growth in synthetic media containing carbohydrates described above.

Anaerobically no acid or gas from glucose and lactose.

Reducing substance is not produced from gluconate.

Glucose, gluconate, 2-keto-gluconate, 5-keto-gluconate, citrate, succinate, ethanol, phenol, benzoate, salicylate, *m*-hydroxy-benzoate, *p*-hydroxy-benzoate, protocatechuate, gentisate, and *p*-amino-benzoate are not utilized as a sole source of carbon.

Sodium chloride of 0.3 to 5.0% is required for growth. No growth in media containing over 9.0%.

Cellulose not attacked.

Diffusible or water-insoluble pigment is not produced.

Optimum temperature: 25° to 30°. Growth scanty at 37°, and no

growth at 42°.

Cytochrome oxidase: Negative.

Catalase: Positive (weak).

Aerobic.

Source: Oil-brines in Japan, 1957.

2. *Pseudomonas stutzeri* (LEHMANN and NEUMANN) KLUYVER

Strains: Nos. 21, 37, 39, 43, 44, 48, 60, 61, 70, and 94.

The strains isolated grew anaerobically by performance of nitrate respiration, hydrolyzed starch, and developed in synthetic media containing starch but did not produce acid. The wrinkled appearance on nutrient agar and glutamate agar plates was characteristic, but the strains forming smooth colonies were also found. The existence of such two morphotypes is ascribed to dissociation, which was already reported by the other investigator (14). Generally, the strains forming wrinkled colonies produced acid from xylose and glucose, but ones forming smooth colonies did not produce acid from carbohydrates. The ability to produce acid from carbohydrates by this species is different from the description which was made by VAN NIEL and ALLEN (14). It is of interest, comparing with the other pseudomonads, that the strains of this species hydrolyze starch and grow in synthetic media containing starch without production of acid. The ability to develop at 37° is also unique property among pseudomonads. This species belongs to the achromogenic group of genus *Pseudomonas*. (10, 11).

Rods, 0.6 to 0.8 by 1.4 to 2.0 microns. Motile with a polar flagellum. Gram-negative.

Nutrient agar colonies: Circular, rough, verrucose to rugose, flat to raised, erose, dull or glistening, pale yellowish brown, opalescent, butyrous to slightly membranous. (Variation: Smooth, entire to slightly lobate, orange brown.).

Glutamate agar colonies: Irregular, bullate, raised, undulate, dull, pale yellowish gray, hard membranous, strongly adherent to medium, medium unchanged. (Variation: Circular, rough, entire to lobate, finely pitted surface, pale yellow, butyrous).

Nutrient agar slant: Growth moderate, filiform, dull, finely pitted surface to wrinkled, pale brownish yellow. (Variation: Glistening, smooth, pale reddish brown to pale brown).

Glutamate agar slant: Growth abundant, filiform, dull, depressed surface to wrinkled, pale yellow, medium unchanged. (Variation: Glistening, pale yellowish white, smooth).

Nutrient broth: Flocculent or wrinkled pellicle, moderately or slightly turbid, moderate sediment. (Variation: Ring or thin pellicle, strong turbid).

Glutamate broth: Flocculent or wrinkled pellicle, almost clear, scant

sediment. (Variation: Thin pellicle, moderately turbid, moderate sediment).

Nutrient gelatin stab: No liquefaction.

Milk: Unchanged.

Litmus milk: Alkaline, litmus reduced, not peptonized.

B. C. P. milk: Alkaline, not peptonized.

Nitrates are reduced to nitrites and gaseous nitrogenous compounds in nitrate broth and succinate-nitrate broth.

Nitrate respiration: Positive.

Indole not produced.

Hydrogen sulfide not produced.

Starch hydrolyzed.

Acetylmethyl carbinol not produced.

Several strains produce acid but no gas from xylose and glucose, yet afford no acid from glycerol, sucrose, lactose and starch in peptone media and in synthetic media. Generally, the strains forming rough colonies produce acid from xylose and glucose, but the strains forming smooth colonies do not produce acid from carbohydrates.

Anaerobically no acid or gas from glucose and lactose.

Reducing substance is not produced from gluconate.

Citrate and succinate are utilized as a sole source of carbon, but glucose, gluconate, 2-keto-gluconate, 5-keto-gluconate, ethanol, phenol, benzoate, *m*-hydroxybenzoate, *p*-hydroxy-benzoate, protocatechuate, gentisate, anthranilate, and *p*-amino-benzoate are not utilized.

Cellulose not attacked.

Diffusible or water-insoluble pigment is not produced.

Optimum temperature: 30° to 37°. Scanty or no growth at 42°.

Cytochrome oxidase: Positive (moderate).

Catalase: Positive.

Aerobic.

Source: Oil-brines and soils in petroleum zone in Japan, 1957.

3. *Pseudomonas putrefaciens* (DERBY and HAMMER) LONG and HAMMER

Strains: Nos. 63 and 64

The strains isolated formed reddish brown colonies on nutrient agar, and rapidly liquefied gelatin and milk. They abundantly produced hydrogen sulfide. This species belongs to the achromogenic group of genus *Pseudomonas* (10, 11).

Rods, 0.6 to 0.8 by 1.4 to 2.4 microns. Occurring in pairs. Motile with a polar flagellum. Gram-negative.

Nutrient agar colonies: Circular, smooth, entire, convex, glistening, pale salmon color to reddish brown, opaque, butyrous.

Glutamate agar colonies: Circular, smooth, entire, umblicate, glistening, pale salmon color to pale pinkish gray, butyrous, medium unchanged.

Nutrient agar slant: Growth abundant, filiform, glistening, thick,

salmon pink color to reddish brown, medium unchanged.

Glutamate agar slant: Growth moderate, filiform, glistening, pale reddish brown, medium unchanged.

Nutrient broth: Ring or very thin pellicle, moderately to strongly turbid, pulverous sediment.

Glutamate broth: Ring, moderately turbid, pulverous sediment.

Nutrient gelatin stab: Rapid liquefaction.

Milk: Peptonized.

Litmus milk: Alkaline, litmus reduced, peptonized.

B. C. P. milk: Alkaline, peptonized.

Nitrite is not produced from nitrate in nitrate broth, but produced in succinate-nitrate broth.

Nitrate respiration: Negative.

Indole not produced.

Hydrogen sulfide abundantly produced.

Starch very slightly hydrolyzed.

Acetylmethyl carbinol not produced.

No acid or gas from glycerol, xylose, glucose, sucrose, lactose and starch in peptone media and in synthetic media.

Anaerobically no acid or gas from glucose and lactose.

Reducing substance is not produced from gluconate.

Glucose, citrate and succinate are utilized as a sole source of carbon, but gluconate, 2-keto-gluconate, 5-keto-gluconate, ethanol, phenol, benzoate, salicylate, *m*-hydroxy-benzoate, *p*-hydroxy-benzoate, protocatechuate, gentisate, anthranilate, and *p*-amino-benzoate are not utilized.

Cellulose not attacked.

Diffusible pigment is not produced.

Optimum temperature: 30°. Moderate growth at 37°, and no growth at 42°.

Cytochrome oxidase: Negative.

Catalase: Positive.

Aerobic.

Source: Oil-brines in Japan, 1957.

4. *Pseudomonas azotoformans* IIZUKA and KOMAGATA

Strains: Nos. 27, 36, 41, 45, 65, 71, and 69.

The strains belonging to this species were abundantly isolated from oil-brines. The isolates grew anaerobically by performance of nitrate respiration. The detail characteristics were similar to those of the original description which was reported by the present authors (15). However, the strain No. 69 was non-motile variety of this species. This species belongs to the fluorescent group of genus *Pseudomonas* (10, 11).

Fig. 1. Micrographs of pseudomonads

1. <i>Ps. iners</i> nov. sp. No. 52	4. <i>Ps. azotoformans</i> No. 45
2. <i>Ps. stutzeri</i> No. 39	5. <i>Ps. schuylkilliensis</i> No. 35
3. <i>Ps. putrefaciens</i> No. 63	6. <i>Ps. aeruginosa</i> No. 2560

Each strain was cultured on nutrient agar slant for 18-24 hr at 20°. Stained by TODA's method.

Fig. 2. Colony of *Ps. stutzeri* No. 37 cultured on glutamate agar plate for 7 days at 30°.

5. *Pseudomonas nitroreducens* IIZUKA and KOMAGATA

Strains: Nos. 23, 40, and 58.

The isolates grew anaerobically by performance of nitrate respiration, and did not liquefy gelatin. The taxonomical characteristics of the isolates were similar to those of the original description which was reported by the present authors (3). This species belongs to the fluorescent group of genus *Pseudomonas* (10, 11).

6. *Pseudomonas schuyllkilliensis* CHESTER

Strains: Nos. 29, 35, 42, and 108

A large number of strains belonging to this species were isolated from oil-brines and the other materials. They did not reduce nitrate to nitrite in nitrate broth, and failed to grow anaerobically by performance of nitrate respiration. The taxonomical characteristics of the isolates were similar to those reported by the present authors (10, 11, 16). This species belongs to the fluorescent group of genus *Pseudomonas* (10, 11).

7. *Pseudomonas aeruginosa* (SCHROETER) MIGULA

Strains: Nos. 2560, 2580, 2600, and 2620.

The strains of this species were not usually found in oil-brines and the other related materials, although this species is commonly found in nature. The isolates grew anaerobically by performance of nitrate respiration, and developed at 42°. This species belongs to the fluorescent group of genus *Pseudomonas* (10, 11).

DISCUSSION

In the course of determinative studies of microorganisms which were isolated from the materials obtained in petroleum zones in Japan, we have found a large number of pseudomonads in oil-brines brought from the oil-layers from two hundred to seven hundred meters beneath. These pseudomonads possess adequate characteristics to exist in oil-layers, although it is considered that the temperature in oil-layers is higher than in the usual habitats of microorganisms. Traces of oxygen are present for permission of aerobic growth of microorganisms. *Ps. stutzeri*, *Ps. azotoformans*, *Ps. nitroreducens*, and *Ps. aeruginosa* can grow anaerobically by performance of nitrate respiration, and *Ps. stutzeri* and *Ps. aeruginosa* can develop at 37°. *Ps. iners* requires a small amount of sodium chloride for growth, and its optimum concentration is very similar to those of oil-brines. With such physiological and biochemical characteristics of the pseudomonads, the reason for their ability to develop in special environments like oil-brines would be found, and therefore, these pseudomonads may play important roles forming the main members of microflora of petroleum zone. In the ecological studies of petroleum zone, one should investigate not only special microorganisms such as hydrocarbon-utilizing bacteria, but also other microorganisms which appear in ordinary media and possess the adaptive characteristics to develop in a limited environment.

SUMMARY

Determinative studies were carried out with the pseudomonads isolated from oil-brines and the other related materials obtained in petroleum zones in Japan, and the following species were identified: *Pseudomonas iners* nov. sp., 2 strains; *Pseudomonas stutzeri* (LEHMANN and NEUMANN) KLUYVER, 10 strains; *Pseudomonas putrefaciens* (DERBY and HAMMER) LONG and HAMMER, 2 strains; *Pseudomonas azotoformans* IIZUKA and KOMAGATA, 7 strains; *Pseudomonas nitroreducens* IIZUKA and KOMAGATA, 3 strains; *Pseudomonas schuylkilliensis* CHESTER, 4 strains; and *Pseudomonas aeruginosa* (SCHROETER) MIGULA, 4 strains. From the physiological and biochemical characteristics of these pseudomonads, the authors discussed the reason for their occurrence in oil-brines.

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