Selection of the Nitrite-Accumulating Bacteria by a Plating Method

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ABSTRACT

A plating method by using a nitrite-sensitive pseudomonad as indicatory cell was established for the selection of the nitrite-accumulating bacteria. The population of these bacteria in paddyfield water, pond or ditch is usually in a range from 2×10^2/ml to 5×10^3/ml. Many of them were identified as *Enterobacter*, *Salmonella*, *Klebsilla*, *Proteus*, *Serratia*, *Escherichia* or *Citrobacter*. The result suggested that the enteric rods having a higher potential to accumulate nitrite than the other bacteria.

INTRODUCTION

Bacteria having nitrate reduction activity can be grouped into three types (Payne, 1973): (1) a complete assimilatory reduction of nitrate to ammonia; (2) an incomplete dissimilatory reduction and accumulation of nitrite in the medium; (3) denitrification or a reduction of nitrate to nitric oxide or nitrogen gas. The reduction of nitrate to nitrite is the key step for the nitrate reduction. In nature, nitrite formed usually was not accumulated because it could be further metabolized. However, under certain circumstances, formation of nitrite may be more rapid than its further transformation, and then accumulated (Alexander, 1973). The accumulation of nitrite in nature caused hazards to humans, animals and plants. Nitrite is also a potential dangeous compound to our health because it may form nitrosoamine, a super-carcinogenic compound, in the presence of secondary amine (Lijinsky and Epstein, 1970).

There are many species of bacteria, including in more than fourty genera, have been known to reduce nitrate. But the activity and their significance in responce to the nitrate reduction in nature is different from species to species. In order to know the ecological impact of these bacteria, it is important to understand their population under different environmental conditions. In our previous study, a pseudomonad very sensitive to nitrite was isolated (Huang and Chang, 1978). In this paper, we reported a plating method by using this isolate as indicator for the assay of the nitrite-accumulating bacteria present in nature environments.

MATERIALS AND METHOD

Source and identification of the indicatory bacterium:

The nitrite-sensitive pseudomomad was isolated from the stem of healthy rice plant in this laboratory. The bacterial morphology was examined under electron microscope. Ultilization of carbohydrate was studied according to the method described by Hugh and Leifson (1953). Formation of pigments was examined by using the media of King *et al.* (1954). Production of fluorescent pigment was examined by UV light in the dark. Biochemical tests including hydrolysis of starch and gelatin;

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activities of oxidase, catalase and urease; nitrate reduction; ammonification and indole production from peptone; Voges-Proskauer (V-P) test and methy red (MR) reaction were tested. The activity of oxidase was studied by the modified method of Stainer *et al.* (1966). Base composition of DNA was determined according to the method of Marmur and Doty (1962). The purified DNA used in this study has a A_{260}/A_{280} ratio of 1.93.

Media:

Nutrient medium containing 0.5% peptone, 0.5% yeast extract, 0.1% glucose, and 0.5% K_2HPO_4 was used to maintain and cultivate bacteria isolated in this study.

Synthetic medium containing 0.01% K_2HPO_4, 0.09% KH_2PO_4, 0.1% $(NH_4)_2SO_4$, 0.025% $MgSO_4 \cdot 7H_2O$, 0.0005% $FeCl_3$ and 1% sodium citrate was used as the basal medium for stydying the nutritional requirement of the indicatory bacterium.

Potato-sucrose (PS) medium containing potato-extract (200 g/l), 1.5% sucrose, 0.2% Na_2HPO_4, 0.05% $Ca(NO_3)_2$, pH 7.2, was used as the assay medium for the isolation of the nitrite-accumulating bacteria.

The plating method for the isolation of the nitrite-accumulating bacteria:

The double-layer plating technique with the nitrite-sensitive pseudomonad as the indicatory bacterium was used for the selection of the nitrite-accumulating bacteria. The bottom (hard) layer was 20 ml PS medium in the presence of 1.5% agar, the top (soft) layer was 4 ml PS with 0.75% agar. The indicator was prepared by cultivating the pseudomomad in nutrient broth for 16 hours at 30°C. Samples for the nitrite-accumulating bacteria were properly diluted before plating. The assay was performed by mixing 0.2 ml of the diluted sample and 0.1 ml of the pseudomonad's culture (the bacterial concentration about 5×10^8/ml) into the melted soft agar which was maintained at 50°C with water bath. The mixture was immediately poured on the hard agar layer and then incubated at 30°C after the agar hardened. The result was examined after 16 hours of incubation. The colony developed on the plate with an inhibition zone against the indicatory bacteria was selected, purified, and then characterized.

RESULTS

The indicatory bacterium used in the plating assay is an aerobic, polar flagellated, Gram-negative rod identified as a species of *Pseudomonas*. Properties and morphology of the pseudomomad was presented in Table 1 and Fig. 1.

The double-layer plating method with the nitrite-sensitive pseudomonad as indicator was used to assay the nitrite-accumulating bacteria present in various samples. Since the pseudomonad was very sensitive to nitrite, the nitrite accumulated by the bacteria present in the tested sample would inhibite the growth of the indicatory cell on the plate. As indicated in Fig. 2, colonies with clear inhibitory zone against the bacterial lawn on plate could be easily enumerated. Population of the nitrite-accumulated bacteria present in the paddy fields, ponds, or ditches were studied by the plating method. As indicated in Table 2, about 1% of the total saprophytic bacteria had the nitrite-accumulating activity.

Twenty-five colonies with positive inhibitory zone were randomly chosen and then isolated. Properties of these isolates were characterized after they were purified by single colony isolation (Table 3). All of them were identified as enteric rods. Among them, 16 isolates were identified as *Enterobacter*, 6 isolates belong to *Salmonella*, *Klebsilla*, *Proteus*,

Table 1. Properties of the indicatory bacterium

Cytological properties and growth characteristics:

Aerobic rod; non-spore forming; Gram-negative; polar flagella.

Colony: round, smooth with light yellow color.

Water soluble pigment: small amount of yellow-green pigment, but no fluorecent pigment.

Cellular organic reserve: poly-β-hydroxybutyrate accumulated.

Pellicle: pellicle adhereed on the flask of liquid culture.

Initial pH range for starting growth: 3.5 to 9.0.

Temperature for maintaining normal growth: 25° to 40°C.

Capacity of salt tolence: No growth above 4%.

Nutritional requirement:

Requiring no growth factor; using ammonium, but not urea or nitrate, as sole nitrogen source; using arginine, many organic compounds as sole carbon source:

Good sole carbon source: glucose, sucrose, lactose, mannose, D-xylose, L-arabinose, D(+)-melibiose, D(+)-cellobinose, trehalose, raffinose, acetate, butyrate lactate, lactate.

Poor sole carbon source: L-xylose, D-ribose, I-inositol, dulcitol, sorbitol, galactose, maltose, rhamnose.

Can not be used as sole carbon source: L-sorbose, mannitol, inuline, fructose, I-erythritol, melezitose.

Biochemical properties:

Positive in the following tests:

Gelatin hydrolysis; ammonification; catalase.

Negative in the following reastiona:

Starch hydrolysis,; nitrate reduction; indole production; M-R test; V-P test; urease; oxidase.

Base composition:

Molar ratio of G+C is 68% (based on *Tm* Vlaue).

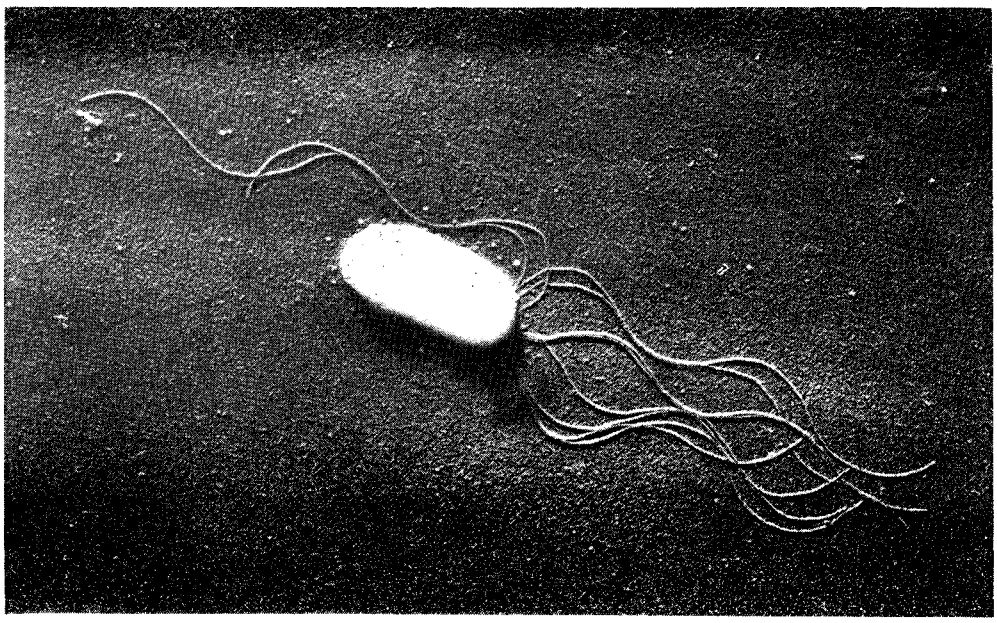

Fig. 1. Morphology of the pseudomonad used as the indicator in the plating method. The picture was taken by electron microscope: 14400×.

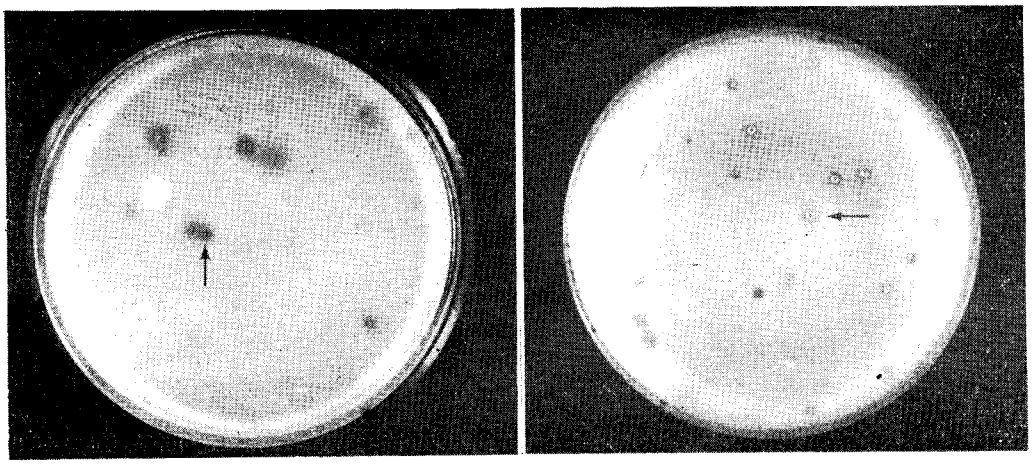

Fig. 2. The bioassay for the selection of the nitrite-accumulating bacteria present in various environments. The bacteria accumulating nitrite on the assay condition could form a inhibitory zone (indicated by the arrow) against the bacterial lawn on the plate. Left picture: a sample taken from paddy field; Right picture; a sample taken from pond B (to see Table 2).

Table 2. Population of the total saprophytic bacteria and the nitrite-accumulating bacteria in certain natural environments

Source of Sample [a]	Total Bacterial Concentration/ml. [b]	Total Nitrite-Accumulating Bacteria/ml. [c]
Paddy-field water	6.1×10^{5}	3.1×10^{2}
Pond A	2.6×10^{6}	1.1×10^{3}
Pond B	7.2×10^{5}	2.9×10^{3}
Ditch A	3.3×10^{6}	5.2×10^{3}
Ditch B	3.2×10^{6}	2.3×10^{2}

(a) Samples were taken from Nankang area, Taipei.
(b) Enumberation by the colony-formation on nutrient agar.
(c) Determination by the plating method described in the paper.

Serratia, *Escherichia* and *Citrobacter* respectively. Three of them required further identification. These 25 isolates were demonstracted to reduce nitrate and accumute nitrite in the presence of nitrate.

DISCUSSION

Based on the study, the plating method is specific for the selection of the nitrite-accumulating bacteria. However, in the assay condition, the formation of inhibitory zone against the indicator required the accumulation of certain amount of nitrite (Huang and Chang, 1978). The bacteria which accumulated nitrite, but could not fulfill the required concentration, would not be able to form the inhibition zone. So the method actually selected only the bacteria which having strong nitrite-accumulating activity. The result of this experience revealed that most of the bacteria with clear inhibition zone were enteric rods. It suggested that the enteric bacteria acoumulated more nitrite than other bacteria under the experimental condition.

Nitrite is an intermediate in nitrification or in denitrification in the natural ecosystem. In nature, this anion does not accumulate because the rate of formation is usually less than the rate of its further metabolism. But under certain circumstances, such as in the soil with high pH and where free ammonia is present, or in the place where the reaction of nitrification or denitrification is not existed, the nitrite produced may be built up. *Escherichia*, *Proteus*, *Salmonella* or other enteric rods are the major

Table 3. Identification of the 25 isolates which forming inhibition zone against the bacterial lawn on the bioassay plates

Reaction tested	Number among the 25 isolates									
	16/25	1/25	1/25	1/25	1/25	1/25	1/25	1/25	1/25	1/25
Glucose ultilization	AG	AG	AG	A	AG	AG	A	AG	AG	AG
Gelatin hydrolysis	−	−	−	−	−	−	−	−	−	−
Starch hydrolysis	−	−	−	−	−	−	−	−	−	−
Nitrate reduced to nitrite	+	+	+	+	+	+	+	+	+	+
Reduction of nitrite	−	−	−	−	−	−	−	−	−	−
Growth in KCN	+	−	+	+	+	+	+	−	+	−
Citrate as sole carbon source	+	−	+	+	+	+	+	+	+	+
M-R test	−	+	+	+	+	−	−	−	−	−
V-P test	−	−	−	+	−	−	+	−	+	+
H_2S production	−	−	+	−	−	−	−	−	−	−
Indole formation	−	+	−	−	−	−	−	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	+
Urease	+	−	−	−	−	+	+	−	+	+
Oxidase	−	−	−	−	−	−	−	−	−	−
Taxonomic position	Enterobacter	Escherichia	Salmonella	Proteus	Citrobacter	Klebsiella	Serratia	uncertain	uncertain	uncertain

(a) A: Acid formed; G: Gas produced; AG: Acid and gas formed.

bacterial population in the microbial flora in our digestive system, and the nitrification or the denitrification reaction usually does not happen in digestive tract. So the nitrite produced by the enteric rods may be accumulated and then pose a potential hazard to our health.

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藉固體培養法篩選「亞硝酸產生細菌」之研究

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摘 要

利用對亞硝酸特別敏感之假單胞桿菌菌株，以固體培養皿法，從事亞硝酸產生細菌的篩選研究。根據結果，水田、池塘以及水溝的樣品中，每毫升約含有 2×10^2 到 5×10^3 的亞硝酸產生細菌，其中大多數屬於腸內桿菌，此結果顯示腸內桿菌產生亞硝酸的活性強於其他細菌。