Isolation and Identification of Respiratory Nitrate Reductase-Producing Bacteria from Soil and Production of the Enzyme

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A bacterial strain MY-12, isolated from rice field soil, and identified as *Enterobacter cloacae*, produced much nitrate reductase in cells in anaerobic culture. Tests were conducted on the growth conditions for anaerobic production of the enzyme. Nitrate was essential for enzyme formation, and iron ions promoted enzyme production. The addition of peptone (2 g/liter) to the synthetic medium increased enzyme production with an increase in growth. The specific activity of the enzyme of this strain, cultured under optimal growth conditions, was higher than that of enzyme of *Escherichia coli*, *Klebsiella aerogenes*, *Bacillus licheniformis* or *Micrococcus denitrificans*. Nitrate reductase of *E. cloacae* MY-12 produced in anaerobic culture was membrane-bound. Some properties of the solubilized enzyme are described.

Respiratory nitrate reductase (EC, 1.9.6.1) is distributed in various species of bacteria.¹⁾ The enzyme is formed in cells when they are cultured anaerobically in the presence of nitrate. This is membrane-associated enzyme and it mediates the transfer of electrons from cytochromes to nitrate which acts as a terminal electron acceptor.²⁾ In some bacteria, the enzyme was solubilized from particulate fraction by heat treatment or the use of detergents and purified to homogeneity.^{2~6)} The molecular,⁷⁾ chemical⁶⁾ and catalytic⁸⁾ properties of the enzyme have been investigated.

In order to characterize respiratory nitrate reductase in other species of facultative bacteria in rice fields, farms, forests and river beds, we attempted to isolate strains with high enzyme yield. Three strains with high yield were isolated from 120 sources. Strain MY-12 produced the most enzyme of three in anaerobic culture and was used for the present study.

The report describes the isolation of the bacteria producing respiratory nitrate reductase, the identification of the strain, growth conditions for enzyme production and proper-

ties of the enzyme.

MATERIALS AND METHODS

Materials. Lysozyme, RNase and DNase were purchased from Seikagaku Kogyo Co., Tokyo.

Strains. *E. cloacae* IFO 12935 was obtained from the Institute for Fermentation, Osaka.

Soil samples. Soils of rice fields, farms, forests and river beds in Hyogo Prefecture were used for the screening tests.

Isolation of microorganisms. The basal medium for screening consisted of the following per liter: $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 15 g; KH_2PO_4 , 3 g; glucose, 4 g; NaNO_3 , 1 g; NH_4Cl , 0.1 g; NaCl , 0.5 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g; and $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 2 mg; pH 7.1. A small amount of NH_4Cl (one-tenth of NaNO_3) was added to the medium for increasing the growth of microorganisms. About 1 g of soil was suspended in 10 ml of 0.8% sterile saline solution. After shaking vigorously for 1 min, 1 drop of the supernatant was applied to a plate on which basal medium containing 15 g/liter of agar was layered, and the plate was placed at 30°C for 3 to 7 days. Grown organisms were transferred to a tube containing basal medium and cultured statically at 30°C for 24 hr. Well-grown organisms were selected and cultured anaerobically in a rubber-stoppered shaking flask filled with 600 ml of medium for 24 hr. Cells were collected by centrifugation at

9000 $\times g$ for 20 min and disrupted with a Kubota 200 M ultrasonic oscillator at 160 W for 5 min. Nitrate reductase activity was then assayed. Organisms with high enzyme productivity were selected.

Enzyme assay. Nitrate reductase was assayed by measuring the nitrite liberated by reduction of nitrate with methyl viologen as an electron donor.⁹⁾ The enzyme reaction mixture consisting of 0.5 ml of 0.1 M K-Na phosphate buffer, pH 7.1, 0.2 ml of 0.2 M NaNO₃ and 0.025% methyl viologen, and 0.3 ml of enzyme solution was put into a small test tube (9 $\times$ 120 mm) with a rubber stopper and preincubated at 30°C for 3 min. The tube was flushed with N₂ to remove the O₂. To start the reaction, 0.1 ml of 0.4% Na₂S₂O₄ prepared freshly in 0.4% NaHCO₃ was added to the mixture. The tube was shaken gently till the mixture turned blue. After incubation for 5 min at 30°C, the stopper was removed and the mixture was stirred vigorously with a tube mixer to oxidize the reduced methyl viologen and remaining Na₂S₂O₄. For determination of nitrite,¹⁰⁾ 0.5 ml of 1% sulfanilamide in 9% HCl and 0.5 ml of 0.02% *N*-1-naphthylethylenediamine dihydrochloride were added to the mixture. After incubation for 10 min at room temperature, 1 ml of deionized water was added, and the absorbance at 540 nm was read. One unit of enzyme activity was defined as the production of 1 μ mol of nitrite per hr at 30°C. Specific activity was expressed in units per mg of protein. Nitrite reductase was assayed by the same method as described above in the reaction mixture containing NaNO₂ as an electron acceptor instead of NaNO₃.¹¹⁾

Protein assay. Protein was determined by the method of Lowry *et al.*¹²⁾

Identification of isolated strain. The isolated bacterium was identified according to "Bergey's Manual of Determinative Bacteriology," 8th ed.¹³⁾ and the method reported by Sakazaki.¹⁴⁾

Growth conditions for enzyme production. For nitrate reductase production, two different anaerobic cultures were carried out. First, a rubber-stoppered shaking flask was filled with 600 ml of medium and flushed with N₂. Before inoculation, 20 mg of Na₂S₂O₄ was added to the medium to completely exclude O₂.¹⁵⁾ Second, the rubber-stoppered shaking flask was filled with 600 ml of medium which was rapidly cooled after sterilization, and the strain was inoculated without flushing with N₂ or Na₂S₂O₄. No differences in cell growth and enzyme production were observed between the two anaerobic cultures. The second method was employed in the following experiments. After the bacterium was precultured statically in 10 ml of medium at 30°C for 8 hr, it was transferred to a flask and incubated at 30°C for 13 hr. Cells were collected by centrifugation at 9000 $\times g$ for 20 min, washed with 0.1 M K-Na phosphate buffer, pH 7.1, and centrifuged again at

17,000 $\times g$ for 20 min. Wet cells were suspended in 0.1 M K-Na phosphate buffer, pH 7.1 (1 g wet cell/10 ml), and disrupted with the ultrasonic oscillator at 160 W for 5 min. The cell-free extract was assayed for nitrate and nitrite reductases. The nitrate reductase activity in the cell-free extract was modified by adding the amount of nitrite which was decreased by nitrite reductase in the extract.

Fractionation of cell-free extract. The bacterium cultured anaerobically was incubated with a lysozyme solution containing 100 mg of lysozyme, 1.5 mg of DNase and RNase and 5 mM EDTA in 100 ml of 0.1 M Tris-HCl buffer, pH 8.0, at 30°C for 30 min and sonicated at 60 W for 1 min. The whole cells were removed by centrifugation at 3000 $\times g$ for 10 min. The supernatant was centrifuged at 40,000 $\times g$ for 2 hr. Nitrate reductase activity was determined in the supernatant and precipitated fractions.

Solubilization of enzyme. The cell-free extract of the bacterium was prepared as described above. After the whole cells were removed by centrifugation at 3000 $\times g$ for 10 min, the supernatant was centrifuged at 40,000 $\times g$ for 1 hr. The resulting precipitated fractions were washed two times by homogenizing the pellet in 0.1 M Tris-HCl, pH 8.8, containing 10% (w/v) sucrose and centrifuging at 40,000 $\times g$ for 1 hr. The final pellet was suspended in the same buffer containing 10% sucrose at a protein concentration of 3.5 mg/ml and heated at 60°C for 20 min. The heated suspension was cooled and centrifuged at 40,000 $\times g$ for 2 hr. From the final pellet, 43% of its enzyme activity was recovered in the supernatant. The solubilized enzyme was used for experiments on catalytic properties of the enzyme.

RESULTS AND DISCUSSION

Isolation of bacteria

From 120 soil samples, 90 strains appeared on agar plates, which could assimilate nitrate as a nitrogen source. Thirteen strains grew in static culture in test tubes, and all strains accumulated nitrite in the medium. The observations suggest that these strains produced nitrate reductase in static culture. The organisms were anaerobically cultured in the shaking flask completely filled with medium, and the nitrate reductase activity of cells was assayed. Strains MY-12, MY-30 and MY-62, all isolated from soils of rice field, grew well (over 1.5 g wet cell/liter) and produced the enzyme in high quantity (over 3000 units/liter). Strain MY-12 produced the most enzyme of three strains and was used for the following

experiments.

Identification of strain MY-12

Strain MY-12 was subjected to identification to determine the taxonomical position of nitrate reductase-producing microorganism. The morphological and physiological properties of the strain are listed in Table I. The strain was a Gram-negative rod and fermentative in O-F test, and made TSI agar yellow. The results show that the strain belongs to Enterobacteriaceae. The physiological tests of the strain was further examined, as contrasted with cultures belonging to the family. Strain MY-12 closely resembled *E. cloacae* IFO 12935 in gas production from various sugars except lactose, decarboxylase activities for lysine, arginine and ornithine, and other physiological properties. Strain MY-12 produced gas rapidly from lactose, whereas gas production from the sugar in *E. cloacae* IFO 12935 was very slow. The isolated strain MY-12 was thus identified

TABLE I. MORPHOLOGICAL AND PHYSIOLOGICAL PROPERTIES OF STRAIN MY-12

Rods, 0.5 to 1.0 by 1.5 to 2.5 microns. Motile with peritrichous flagella. Gram-negative.
Nutrient agar colonies: Circular, smooth, entire, flat to convex, yellowish white, glistening, butyrous, transparent, growth abundant.
Nutrient agar slant: Spreading, yellowish white, glistening, butyrous, medium unchanged, transparent, growth abundant.
Nutrient broth: Turbid with sediment.
Gelatin stab: Liquefaction, growth abundant.
Litmus milk: Acid, not decolorized, coagulated.
B.C.P. milk: Acid, coagulated.
TSI agar: Yellow, gas production, hydrogen sulfide not formed.
O-F test: Fermentative. Methyl red test: Negative.
V.P. test: Positive. I.P.A. test: Negative.
Cytochrome oxidase: Negative. Catalase: Positive.
Ureas: Positive. Citrate and acetate utilized.
Gas from glucose, sorbitol, rhamnose, cellobiose, arabinose, raffinose and lactose.
No gas from starch, glycerol and inositol.
Arginine and ornithine decarboxylases: Positive.
Lysine decarboxylase: Negative.
Good growth at 20°C to 45°C. Good growth at pH 4.5 to pH 9.5.
Facultatively anaerobic.

as *E. cloacae* (Jordan) Hormaeche and Edwards.

Growth conditions for enzyme production

Effects of metals. Table II shows that the addition of Fe^{2+} to the medium promoted cell growth and enzyme production. The total enzyme activity in cells cultured in basal medium containing 5 mg/liter of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ without molybdate was about 8 times that in cells cultured without these metals. The supply of molybdate to the medium, in addition of Fe^{2+} further promoted enzyme production. In the following tests for growth conditions, 5 mg/liter of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ was supplied to the basal medium. Iron and molybdenum were reported to exist in respiratory nitrate reductase of *E. coli*⁷⁾ and *B. licheniformis*.⁶⁾ The enzyme of *E. cloacae* MY-12 also seems to have these metals.

Effects of C- and N-sources. The amounts of NH_4Cl and glucose in medium did not influence enzyme production per cell much, but influenced cell growth. As a result, the proper supply of these compounds increased total enzyme activity.

Effects of natural media. The addition of

TABLE II. EFFECTS OF METALS ON GROWTH AND ENZYME PRODUCTION OF *E. cloacae* MY-12

$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ or $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ or both were added to basal medium.

$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (mg/liter)	$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ (mg/liter)			
	0	2	5	10
0	0.80 ^a	0.89	0.86	0.71
	0.45×10^4 units/liter ^b	0.71	0.70	0.53
2	0.93	1.07	1.09	—
	2.51	2.85	3.03	—
5	1.10	1.10	0.98	—
	3.43	4.16	3.57	—
10	1.07	—	—	—
	2.21	—	—	—

^a Upper row, growth (O.D. at 660 nm).

^b Lower row, total activity.

TABLE III. EFFECTS OF NATURAL MEDIA ON GROWTH AND ENZYME PRODUCTION

Polypepton, and meat extract and/or yeast extract were added to basal medium containing 5 mg/liter of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ and 10 g/liter of glucose.

Natural media (g/liter)		Growth (O.D. at 660 nm)	Total activity (units/liter) $\times 10^4$
Polypepton	0.5	1.26	3.81
	1	1.39	5.10
	2	1.86	9.03
	5	2.24	7.60
	10	2.60	5.77
	20	2.71	4.79
Meat extract	2	1.43	4.39
	5	1.57	5.01
	10	1.77	7.14
	20	2.24	6.43
Yeast extract	0.5	1.45	5.91
	1	1.63	4.94
	2	1.86	5.47
Yeast extract Polypepton	0.5 2	2.04	4.87
	0.5 10	2.56	6.53
Meat extract Polypepton	10 2	2.32	6.16
	10 10	2.63	5.40
Yeast extract Meat extract	0.5 10	2.74	4.10
Polypepton		2	

Polypepton (Daigoeiyo), and meat extract (Riken Vitamin) and/or yeast extract (Daigoeiyo) increased cell growth, but enzyme production varied. The strain produced the most enzyme in medium containing 2 g/liter of Polypepton, as shown in Table III.

Effect of nitrate. Table IV shows the effect of nitrate on cell growth and enzyme production. *E. cloacae* MY-12 grew anaerobically without nitrate in the presence of NH_4Cl or polypepton as a N-source, but nitrate reductase activity in the strain was very low. The addition of 0.5 to 1 g/liter of NaNO_3 increased enzyme production. But growth of the strain was inhibited in medium containing over

TABLE IV. EFFECT OF NaNO_3 ON GROWTH AND ENZYME PRODUCTION

NaNO_3 was added to optimal medium.

NaNO_3 (g/liter)	Growth (O.D. at 660 nm)	Total activity (units/liter)
0 ^a	0.98	0.44×10^4
0	1.49	0.85
0.5	2.09	6.43
1	1.86	9.03
2	1.26	5.98
3	1.01	4.04

^a The sole nitrogen source was 1 g/liter of NH_4Cl without Polypepton.

FIG. 1. Time Course of Growth and Enzyme Production of *E. cloacae* MY-12.

The strain was anaerobically cultured in optimal medium at 30°C.

O—O, growth; Δ — Δ , total activity; $\square$ — $\square$, pH.

2 g/liter of NaNO_3 , although enzyme activity per cell did not decrease so much. In these media, a high concentration of nitrite was detected after incubation for 13 hr, which seemed to inhibit growth of the strain in high concentration of nitrate.

From these growth condition tests, the optimal medium for enzyme production consisted of the following per liter: $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 15 g; KH_2PO_4 , 3 g; glucose, 10 g; NaNO_3 , 1 g; NH_4Cl , 0.1 g; NaCl , 0.5 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 2 mg; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 5 mg; and Polypepton, 2 g; pH 7.1.

Time course of growth and enzyme production

Figure 1 shows that *E. cloacae* MY-12

produced the maximal quantity of enzyme in optimal medium from the end of exponential phase to the origin of stationary phase, that is, after incubation for 12 to 13 hr. The total enzyme activity gradually decreased in the stationary phase, but maintained half the maximal level even after incubation for 24 hr. The pH value of medium was varied from the original pH 7.1 to 6.0.

Comparison of specific activity with other strains

The specific activity of nitrate reductase of *E. cloacae* MY-12 was compared with that of enzymes (respiratory type) reported in other strains. When *E. cloacae* MY-12 accumulated the most enzyme under optimal growth conditions (9.03×10^4 units/liter, see Table III), the specific activity of enzyme in the cell-free extract prepared by disruption with the ultrasonic oscillator was 158 units/mg. The enzyme value was the second highest of six strains listed in Table V, although the enzyme activities of *M. denitrificans* and *Micrococcus halodenitrificans* were assayed at 37°C.

Localization of enzyme in cells

Cell-free extract of *E. cloacae* MY-12 obtained by lysozyme digestion and sonic disruption was fractionated by centrifugation at $40,000 \times g$ for 2 hr, after the whole cells were removed. Eighty-one percent of nitrate reductase was detected in the precipitated fraction. The observation showed that the enzyme formed in anaerobic culture of the strain is principally membrane-bound and seems to belong to the respiratory type.⁹⁾

Optimal pH

Nitrate reductase solubilized from membrane of *E. cloacae* MY-12 had the optimal pH at 7.1 for NO_3^- as an electron acceptor (Fig. 2(a)). The optimal pH of enzymes of *E. coli*²⁾ and *K. aerogenes*⁵⁾ was 7.1 and 6.9, respectively, whereas that of *M. denitrificans*³⁾ and *M. halodenitrificans*¹⁷⁾ was 5.6~

TABLE V. SPECIFIC ACTIVITY OF RESPIRATORY NITRATE REDUCTASE PRODUCED BY VARIOUS STRAINS

Strains	Specific activity (units/mg)	References
<i>E. cloacae</i> MY-12	158	This study
<i>K. aerogenes</i>	40	5
<i>E. coli</i> K-12	100	16
<i>B. licheniformis</i>	14	6
<i>M. denitrificans</i>	75 ^a	3
<i>M. halodenitrificans</i>	250 ^a	17

^a Enzyme activity was assayed at 37°C.

FIG. 2. Optimal pH (a) and pH-Stability (b) of Solubilized Nitrate Reductase.

(a) Optimal pH: The enzyme activity (0.5 units of enzyme) was assayed in buffers of various pH values with ionic strength 0.13 at 30°C. (b) pH-Stability: The enzyme (1.5 units) was incubated in buffers of various pH values with ionic strength 0.2, containing 10% sucrose at 5°C. After 20 hr, the remaining activity was assayed at pH 7.1. The buffers used were: ○—○, $\text{CH}_3\text{COOH}-\text{CH}_3\text{COONa}$; ●—●, $\text{KH}_2\text{PO}_4-\text{Na}_2\text{HPO}_4$; △—△, Tris-HCl; ▲—▲, $\text{NaHCO}_3-\text{Na}_2\text{CO}_3$.

5.8 and 6.3, respectively. From these data, the nitrate reductase of *E. cloacae* MY-12 seems to have a similar optimal pH to *E. coli* and *K. aerogenes* rather than to the two strains belonging to the genus *Micrococcus*.

pH-Stability and thermostability

The solubilized nitrate reductase of *E. cloacae* MY-12 lost about three-fourth of its activity after storage for 24 hr at 5°C in 0.1 M Tris-HCl, pH 8.8, without sucrose. However, the enzyme maintained over 90% of activity after 24-hr storage at 5°C in buffer containing 10% sucrose, so that the pH-stability and thermostability of the enzyme were tested in buffers containing 10%

FIG. 3. Effect of Temperature on Enzyme Activity (a) and Its Thermostability (b).

(a) Effect of temperature: The enzyme activity (0.05 units of enzyme) was assayed at various temperatures and at pH 7.1 in 5-min reaction. (b) Thermostability: The enzyme (2.5 units) was incubated in 0.1 M Tris-HCl buffer, pH 8.8, containing 10% sucrose at various temperatures. After 10 min, the remaining activity was assayed at 30°C and pH 7.1.

sucrose. Figure 2(b) shows that the enzyme was stable in a pH range from 6.5 to 10. The enzyme was stable up to 50°C, and at 60°C about 85% of activity was maintained (Fig. 3(b)). Heat treatment of the enzyme of *E. coli*⁽²⁾ at 60°C in buffer without sucrose was reported to cause no inactivation. These data show that the enzyme of *E. cloacae* MY-12 was somewhat unstable to heat treatment at 60°C compared with *E. coli*, although the enzyme of *E. cloacae* MY-12 was incubated in buffer containing 10% sucrose.

Effect of temperature on enzyme activity

The enzyme of *E. cloacae* MY-12 had maximal activity at 75°C in 5-min reaction, as shown in Fig. 3(a).

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