Growth Yield of a Denitrifying Bacterium, *Pseudomonas denitrificans*, under Aerobic and Denitrifying Conditions

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SUMMARY

The efficiency of denitrification, or anaerobic respiration, in *Pseudomonas denitrificans* was investigated, using growth yield as an index. Glutamate was mainly used as the sole source of energy and carbon. In batch culture, the growth yield per mole of electrons transported through the respiratory system under denitrifying conditions was about half that under aerobic conditions. Similar figures were also obtained in chemostat cultures under glutamate-limited conditions. The decrease in growth yield under denitrifying conditions could be due to the restriction of phosphorylation associated with nitrate reduction to nitrogen gas.

INTRODUCTION

The energy yield of fermentation and respiration has been investigated with a variety of substances in a number of facultatively anaerobic bacteria (Stouthamer, 1969; Forrest, 1969; Payne, 1970). The influence of electron acceptors on the energy yield of respiration has also been examined, using *Aerobacter aerogenes* and *Proteus mirabilis* grown under anaerobic conditions (Hadjipetrou & Stouthamer, 1965; Stouthamer & Bettenhausen, 1972). The metabolism of glucose in these bacteria under anaerobic conditions, however, is mainly fermentative even in the presence of nitrate, and the citric acid cycle does not function to any significant extent. Moreover, the product of nitrate reduction in *A. aerogenes* depends upon its carbon sources; with glucose, nitrate is almost completely reduced to ammonia, but with mannitol, about half of the nitrate is reduced only to nitrite. The situations are thus too complicated for an exact comparison to be made between the energy yield of anaerobic (nitrate) respiration and aerobic respiration.

We attempted to solve this problem using the denitrifying bacterium, *Pseudomonas denitrificans*, which can use nitrate in place of oxygen as a terminal electron acceptor under anaerobic conditions but lacks the capacity for fermentation. The nitrate consumed is quantitatively recovered as nitrogen gas. Under anaerobic conditions, the bacterium cannot grow with glucose and ammonia in the presence of nitrate. If glutamate or some other amino acid is provided together with nitrate, however, the bacterium can grow at an appreciable rate.

We report here, on the growth of *P. denitrificans* in batch culture and energy-limited continuous culture under aerobic and denitrifying conditions, and discuss the difference in efficiency between aerobic and nitrate respiration. Experiments with electron acceptor-limited continuous culture are described in the next paper (Koike & Hattori, 1975).

METHODS

Organism. The *Pseudomonas denitrificans* used was isolated from the bottom mud of Lake Hamana, a brackish lake in Japan, and was kindly identified by Dr Tsuru, Fermentation Research Institute. It was maintained on a peptone-yeast extract agar slant at 4 °C.

Culture media. The complete medium contained (g/l): either brain-heart infusion (Difco), 10, and NaCl, 10; or Polypeptone (Daigo Eiyo Kagaku), 10, yeast extract (Difco), 1, and NaCl, 10. The pH was adjusted to 7.0 before autoclaving. The basal synthetic medium contained (per litre): 10 g NaCl, 1.5 g KH_2PO_4 , 5 g K_2HPO_4 , 5 g KNO_3 , 1 g NH_4Cl , 0.2 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.02 g CaCl_2 , and one drop of a trace metal mixture consisting of 0.5 % (w/v) each of MnCl_2 , CuSO_4 , FeCl_3 and $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$. The phosphates were autoclaved separately from the other mineral salts. The indicated amounts of organic substance were added to the basal medium, and the pH adjusted to 7.1.

In the medium used for continuous culture, NH_4Cl was omitted and sodium citrate, 0.05 mM final concentration, added as a chelating agent.

Measurement of growth yield in batch culture. L-shaped culture tubes (about 50 ml in volume) were used to determine the growth yield. Bacteria collected at the late exponential stage were washed twice with 2 % (w/v) NaCl solution, and inoculated in 8 ml of culture medium at approximately 2 % of the final yield. The culture tubes were continuously shaken at 30 °C. For anaerobic culturing, L-tubes fitted with glass stop-cocks were used. After inoculation, the tubes were evacuated and refilled with oxygen-free argon. This procedure was repeated four times.

Growth was followed by measuring, at regular intervals, the extinctions at 660 nm with a Spectronic 20 spectrophotometer (Bausch and Lomb). Three tubes were used for each experiment and the results averaged. Growth rates were expressed in terms of the reciprocal of the generation time in hours. Yields determined by the E_{660} measurements were expressed in terms of dry weight: an E_{660} of 0.100 corresponded to 68 mg dry wt/l with a maximum variation of 3 %, irrespective of substrates and culture conditions.

Measurement of cell yield in continuous culture. The continuous culture apparatus used was similar to that described by Evans, Herbert & Tempest (1970). Temperature was maintained at 30 ± 0.1 °C. The pH of the culture medium was set at 7.0 ± 0.2 by changing the pH values of the phosphate buffer (0.05 M) in the medium supplied. The medium was fed from a 20 l carboy into the culture vessel (1 l in volume) by a peristaltic pump. To prevent back-contamination in the feed lines, a heating element was placed at the medium inlet as described by Jannasch (1967). The culture vessel was equipped with a flat-blade impeller turning at 720 rev./min. For aerobic culture, water-saturated air was sparged below the impeller at a rate of 250 ml/min. For anaerobic culture, air was replaced with high purity argon (50 ml/min). To minimize foaming, silicon antifoam (Toshiba Denki Co. Ltd) was added at a concentration of 50 p.p.m. The culture usually attained steady state after a period of less than four doubling times when the dilution rate was changed (constant values of cell density were taken as indicating a steady state).

For the determination of cell yield, 150 ml portions of the culture were withdrawn. The bacteria were spun down at 18000 g for 15 min at 4 °C, suspended in distilled water and dried at 105 °C. The supernatant was saved for chemical analysis.

Determination of oxygen consumption and carbon dioxide production. Oxygen consumption during growth was measured manometrically. L-tubes with stop-cocks were used as incubation flasks. A small tube containing 0.5 ml of 40 % KOH was inserted to trap carbon dioxide.

Carbon dioxide production was determined gravimetrically. L-shaped culture tubes with

top-cocks were also used. When growth had ceased, 2 ml of 0.1 N-HCl was carefully added to give a pH of about 3.0. The tube was connected to a vacuum line through two carbon dioxide traps each containing 20 ml of 0.5 N-KOH free of carbonate. When the pressure was reduced to about 40 mmHg, carbon dioxide-free air was introduced. The same procedure was repeated. The CO₂ absorbed in the KOH was converted to BaCO₃ by the addition of 5 ml 0.5 M-BaCl₂; the BaCO₃ was collected on a filter and weighed. The culture immediately after inoculation was used as control. The experimental error was less than 3 %.

Determination of gaseous products. The gaseous products, with the exception of carbon dioxide, were measured by a mass-spectrometric method. A 200 ml syringe was used as the culture vessel. The inoculated culture medium was flushed with high quality argon for 30 min at 30 °C. A 100 ml portion of the inoculated medium was transferred into the syringe without exposure to air, and incubated at 30 °C. Six syringes were set up for the time course experiment. After incubation, 10 ml of the culture medium was removed for the analyses of nitrate and nitrite; the rest of the medium, together with gaseous products, was carefully introduced into a 500 ml reservoir, which had two outlets each with a stop-cock, by replacing the saturated NaCl solution with which the reservoir had been filled. The reservoir was connected to a vacuum line, and the gaseous products, including the dissolved gases, were extracted thoroughly with the aid of a Toepler pump and introduced into a gas reservoir containing 5 ml KOH. After standing for 24 h, during which time the CO₂ was completely absorbed by KOH, the reservoir was connected to the inlet of a Hitachi RMU-6 mass-spectrometer. The mass numbers of 28, 30, 40 and 44 were selected for nitrogen, nitric oxide, argon and nitrous oxide, respectively. The amounts of the individual gases were calculated from the relative height of each mass peak to that of argon. The saturated concentration of argon in the medium was used as reference. The result of a blank test was used to correct for the small amount of nitrogen gas which was inevitably contained in the saturated NaCl solution.

Determination of amino acids, nitrate and nitrite. The concentration of amino acids in the culture medium was determined by the ninhydrin method of Yemm & Cocking (1955). To eliminate ammonia, the samples were brought to pH 12 by adding KOH, and then dried in a desiccator over H₂SO₄. The residual ammonia was less than 1 µM. The concentration of glutamate was enzymically determined, using L-glutamate decarboxylase (Kyowa Hakko Co., Tokyo). The CO₂ liberated was manometrically measured with a Warburg apparatus (Umbreit, Burris & Stauffer, 1964).

The concentrations of nitrate and nitrite were determined by the methods of Wood, Armstrong & Richards (1967) and Bendschneider & Robinson (1952), respectively.

Other analytical procedures. Dissolved organic carbon in the culture medium was determined with an infrared analyser after the wet oxidation method of Menzel & Vaccaro (1966).

Determination of acetate was done by gas chromatography (Packett & McCune, 1965). The elementary composition of cells was determined by a Yanagimoto MT-1 C.H.N. analyser.

RESULTS

Nutritional requirements

All the organic compounds tested, except for mannitol, supported the growth of *P. denitrificans* under aerobic conditions, while only the amino acids and the peptone-yeast extract mixture were effective under both aerobic and denitrifying conditions (Table 1). On the other hand under denitrifying conditions *P. denitrificans* utilized organic acids, such

Fig. 1

Fig. 2

Fig. 1. Changes in nitrate and nitrite concentration and pH in the culture medium during aerobic growth of *P. denitrificans*. Culture conditions: glutamate, 5 mM; KNO₃, 15 mM; 30 °C.

Fig. 2. Changes in nitrate and nitrite concentration and pH in the culture medium during anaerobic growth of *P. denitrificans*. Culture conditions: glutamate, 15 mM; KNO₃, 16 mM; 35 °C.

Table 1. Growth of *P. denitrificans* under aerobic and anaerobic conditions

Except for complex medium, 10 mM of the substrate indicated were added as carbon and energy source. Incubation temperature 30 °C.

Substrate	Growth rate (h ⁻¹)	
	Aerobic	Anaerobic
Glucose	0.41	0.00
Mannitol	0.00	0.00
Lactate	1.1	0.00
Acetate	1.0	0.00
Citrate	0.85	0.00
Succinate	0.64	0.00
Malate	0.71	0.00
Glutamate	0.95	0.20
Glutamate*	0.66	0.14
Aspartate	1.0	0.19
Alanine	0.60	0.085
Peptone-yeast extract*	0.88	0.19

* Incubation temperature 25 °C.

as lactic acid, as electron donors for denitrification. It is suggested that some steps of amino acid formation are blocked in the absence of oxygen. The growth rate under denitrifying conditions was $\frac{1}{5}$ to $\frac{1}{7}$ of that under aerobic conditions. Nitrate was indispensable for the growth of the bacterium under anaerobic conditions.

Reduction of nitrate during growth under aerobic and anaerobic conditions

The concentration of nitrate and nitrite in the culture medium was measured during the growth of *P. denitrificans* under aerobic and anaerobic conditions with glutamate as carbon and energy source. Under aerobic conditions there was no change in the nitrate concentration. The concentration of nitrite increased slightly at the end of the incubation, but was less

Fig. 3

Fig. 4

Fig. 3. The effects of glutamate concentration on the growth rate and the growth yield of *P. denitrificans* under aerobic and denitrifying conditions at 25 °C. (A) denitrifying growth rate; (B) aerobic growth rate; (C) denitrifying growth yield; (D) aerobic growth yield.

Fig. 4. Double-reciprocal plots of Y against μ in aerobic culture and denitrifying culture of *P. denitrificans*. Data given in Table 6 were used. Linear regression analysis was applied for the determination of $Y_{\max}$ and m . ○, Aerobic growth; ●, denitrifying growth.

Table 2. Nitrogen balance during the growth of *P. denitrificans*

Nitrate (15.1 mM) was added as terminal electron acceptor. Glutamate (10 mM) was used as carbon and energy source. Growth was limited by nitrate. Incubation time 42 h. Incubation temperature 30 °C.

NO ₃ ⁻ consumed (mg-atom N)	Nitrogen products (mg-atom N)			
	NO ₂ ⁻	NO	N ₂ O	N ₂
15.1	0.5 × 10 ⁻³	1.7 × 10 ⁻³	0.0 × 10 ⁻³	14.4

than 0.1 % of that of nitrate (Fig. 1). Under anaerobic and nitrate-limited conditions, the growth yield depended on the concentration of nitrate; no growth took place after the exhaustion of nitrate (Fig. 2). Nitrite concentrations in the medium were less than 4 μM throughout the incubation. The nitrate reduced was almost quantitatively recovered as nitrogen gas under denitrifying conditions (Table 2). The amount of nitric oxide produced was only about 0.01 % of that of nitrogen gas (Table 2).

Growth yield in batch culture

The growth rates under aerobic and denitrifying conditions were independent of the concentration of glutamate when it was supplied in concentrations higher than 2 mM (aerobic) and 5 mM (anaerobic), respectively (Fig. 3, lines A and B). On the other hand, the growth yield was proportional to the concentration of added glutamate (Fig. 3, lines C and D). No glutamate was left when the growth stopped. The growth yield per mole of glutamate consumed under denitrifying conditions was approximately half that obtained under aerobic conditions. This was also the case when the bacterium was grown on the other amino acids tested, although the growth yield decreased with decrease in the carbon number of the amino acid (Table 3).

The growth yield per mole of terminal electron acceptor under the conditions of denitrification was also about half of that under aerobic conditions (Table 4). One molecule of oxygen accepts four electrons via the respiratory system, and one molecule of nitrate accepts

Table 3. *Effect of amino acids as the sole source of organic substance on growth yield of P. denitrificans under aerobic and denitrifying conditions at 30 °C*

Substrate	Growth yield (g/mol)		Ratio (%)
	Aerobic	Denitrifying	
Glutamate	64.0	33.3	52.0
Aspartate	36.3	19.3	53.2
Alanine	31.4	12.3	39.2

Table 4. *Growth yield of P. denitrificans under aerobic and denitrifying conditions*

Glutamate (3 and 5 mM) was used as carbon and energy source and the results averaged. Under denitrifying conditions, KNO₃ (40 mM) was added as terminal electron acceptor. Incubation temperature 30 °C.

Growth	Growth yield (g/mol)	
	O ₂ or NO ₃ ⁻	Electron equivalent
Aerobic	34.5	8.63
Denitrifying	18.0	3.60

Table 5. *Carbon balance in growth of P. denitrificans with batch culture*

Incubation temperature 30 °C. Indicated amounts of glutamate, as sole source of carbon and energy, were added in 8 ml culture medium. Growth was limited by glutamate. Figures in parentheses represent the ratios of the carbon products to consumed glutamate on a carbon basis.

Initial glutamate	Carbon (mg)		
	Bacteria	CO ₂	Total (bacteria + CO ₂)
Aerobic growth			
2.40	1.08 (45 %)	1.22 (51 %)	2.30 (96 %)
2.40	1.06 (44 %)	1.31 (55 %)	2.37 (99 %)
1.92	0.86 (45 %)	0.98 (51 %)	1.84 (96 %)
Denitrifying growth			
3.36	0.86 (26 %)	1.74 (52 %)	2.60 (78 %)
3.36	0.85 (25 %)	1.77 (53 %)	2.62 (78 %)
2.40	0.62 (26 %)	1.32 (55 %)	1.94 (81 %)

five electrons when it is reduced to molecular nitrogen. The growth yields, calculated on the basis of electrons transported through the aerobic or denitrifying system, are given in Table 4.

Carbon balance in batch culture

The carbon contents of bacteria grown under the two culture conditions were almost identical (45 % for aerobic and 47 % for denitrifying conditions). When the bacteria were grown with oxygen, 44 to 45 % of the glutamate-carbon was used to produce bacteria and 51 to 55 % was converted to carbon dioxide (Table 5). Within the limits of experimental error, the sum of the two was equal to the amounts of added glutamate-carbon. It is suggested that glutamate is completely oxidized by respiration under aerobic conditions.

The recovery of glutamate-carbon under the conditions of denitrification ranged from 78 to 81 % at the end of growth (Table 5). No glutamate was left in the medium and large amounts of acetate were accumulated. After growth stopped, carbon dioxide production and nitrate consumption continued for about 10 h, without change in the bacterial mass.

Table 6. Growth yield of *P. denitrificans* under aerobic and denitrifying conditions

Glutamate (4 ± 0.1 mM) was added as the limiting substance of continuous culture. Specific growth rate was obtained from the dilution rate. Culture temperature 30 °C.

Aerobic growth			Denitrifying growth		
Specific growth rate (h^{-1})	Growth yield		Specific growth rate (h^{-1})	Growth yield	
	g/mol glutamate	g/electron equiv*		g/mol glutamate	g/electron equiv*
0.092	52.1	6.12	0.046	30.8	3.03
0.10	52.5	6.20	0.056	31.6	3.14
0.11	51.5	5.98	0.062	34.8	3.60
0.15	50.7	5.80	0.072	34.1	3.51
0.21	54.6	6.73	0.074	34.5	3.57
0.24	54.6	6.73	0.085	34.0	3.48
0.27	56.0	7.08	0.096	35.5	3.72
0.33	56.7	7.28			

* Calculated growth yield.

Table 7. Dissolved organic carbon concentrations in the culture medium of *Pseudomonas denitrificans*

Glutamate (4 ± 0.1 mM; 240 ± 6 mg carbon/l) was used as the limiting substance of continuous culture. Culture temperature 30 °C. The specific growth rate was obtained from the dilution rate.

Specific growth rate (h ⁻¹)	Carbon (mg/l)			Residual organic carbon (%)
	Total dissolved	Residual glutamate	Difference	
Aerobic				
0.092	4.8	0.0	4.8	2.0
0.15	4.2	1.2	3.0	1.3
0.33	3.6	1.2	2.4	1.0
Denitrifying				
0.056	6.2	0.0	6.2	2.6
0.085	7.4	2.2	5.2	2.2
0.096	3.9	2.2	1.7	0.7

The final recovery of the carbon was almost 100 %. This can be explained by the shift of the rate-limiting step in the processes of glutamate oxidation caused by the change in terminal electron acceptors. Under anaerobic conditions glutamate serves not only as the source of energy and carbon but also as the source of nitrogen. Glutamate cannot be replaced by ammonia as nitrogen source. It is likely that glutamate, as nitrogen source, limits the anaerobic growth of the bacterium.

Growth yield in continuous culture

Growth yields per mole of glutamate were determined, using a chemostat, under glutamate-limited conditions (Table 6). Glutamate (4 mM) was continuously fed as sole source of energy, carbon and nitrogen. After the establishment of steady state, glutamate concentrations in the chemostat were less than 0.02 mM under aerobic conditions and less than 0.04 mM under denitrifying conditions. When compared at similar specific growth rates, the growth yield under the denitrifying conditions was about 35 % lower than that under aerobic conditions. The amounts of dissolved organic compounds other than glutamate in the

chemostat were about 2 % of initial glutamate or less in terms of carbon (Table 7), suggesting almost complete oxidation of glutamate irrespective of the terminal electron acceptors (oxygen or nitrate). Under denitrifying conditions, nitrate was reduced to nitrogen gas; no intermediary products of nitrate reduction were detected.

Following Johnson (1964), the consumption of oxygen or nitrate in the chemostat was estimated from the consumption of glutamate, the growth yield and the elementary composition of the bacteria, postulating complete oxidation of glutamate. Using the values obtained, the growth yields were calculated on the basis of electrons transported through the respiratory systems (Table 6). The growth yield under denitrifying conditions was about 60 % of that under aerobic conditions.

DISCUSSION

The complete oxidation of glutamate under both aerobic and anaerobic (denitrifying) growth conditions suggests that the metabolic pattern of glutamate degradation in our strain of *P. denitrificans* is unaltered by terminal electron acceptors of respiration. Moreover, the present strain lacks the capacity for fermentation. We can thus estimate the difference in energy yield between aerobic respiration and nitrate respiration on the basis of the data obtained for growth yield. In batch culture, the growth yield per mole of electrons transported through the respiratory system was reduced by about 60 % when the terminal electron acceptor was switched from oxygen to nitrate (Table 4). A similar but somewhat smaller reduction was observed in chemostat culture under glutamate-limited conditions (Table 6).

The growth rate in batch culture under aerobic conditions differed from that under denitrifying conditions by a factor of five (Fig. 3). A transient accumulation of acetate was observed only during growth under denitrifying conditions. It appears that the limiting steps for growth in batch culture are different under these two culture conditions and the values for growth yield obtained in batch cultures should therefore not be compared directly. The data obtained from chemostat culture experiments under glutamate-limited conditions are more satisfactory, because the complete oxidation of glutamate takes place under both aerobic and denitrifying conditions (Table 7). Acetate is not formed as a product of glutamate degradation. We can thus compare the growth yield under growth conditions comparable with each other except for the terminal electron acceptors.

To assess the energy yield from the growth yield, the contribution of maintenance energy to growth yield (Pirt, 1965) and the extent of the energy coupling during growth (Senez, 1962) must be considered. If the energy-yielding metabolism be tightly conjugated with the energy-consuming reactions of biosynthesis, the observed (or apparent) growth yield can be represented, according to Pirt (1965), by the equation,

$$1/Y = m/\mu + 1/Y_{\max},$$

where Y is observed growth yield, $Y_{\max}$ is true growth yield, m is the maintenance energy coefficient, and μ is the specific growth rate. For the purpose of calculation, the growth yield and maintenance energy coefficient are expressed in terms of consumed electron acceptors.

Double reciprocal plots of Y against μ in chemostat cultures under aerobic and denitrifying conditions actually gave straight lines (Fig. 4). On extrapolating to zero ($\mu = \text{infinity}$), $Y_{\max}$ values were estimated to be 4.5 and 7.7 g/electron equivalent for growth under denitrifying and aerobic conditions, respectively, and the respective values for m , estimated from the slopes of these straight lines, were 4.9×10^{-3} and 3.3×10^{-3} electron equivalents/g/h.

The values for $Y_{\max}$ and m in aerobic culture are within the order obtained with other bacteria under energy source-limited conditions (Nagai & Aiba, 1972).

The linear relationship between the reciprocals of Y and μ , and the positive values for $Y_{\max}$ guarantee that the uncoupling between catabolic and anabolic metabolism, if it occurs, is not appreciable under our experimental conditions (cf. Pirt, 1965; Nagai & Aiba, 1972). Under these circumstances, the true growth yield ($Y_{\max}$), or growth yield corrected for maintenance energy, is expected to be proportional to the energy yield. From the ratio of $Y_{\max(\text{nitrate})}$ to $Y_{\max(\text{oxygen})}$ (0.58), we can conclude that nitrate respiration is about 40 % less efficient than aerobic respiration for the biosynthesis of cell materials. A similar relation holds true with respect to the maintenance energy ($m_{\text{oxygen}}/m_{\text{nitrate}} = 0.67$).

Using subcellular preparations of *Micrococcus denitrificans*, John & Whatley (1970) observed that, with NADH as the electron donor, ATP synthesis coupled to the reduction of oxygen is about 70 % more efficient than that coupled to the reduction of nitrate to nitrite. On a thermodynamic basis, the free energy change associated with oxidation of glucose by oxygen is almost identical with that in the oxidation coupled with denitrification (Engel, 1958), as is the oxidation of NADH (26.2 and 24.8 kcal/electron equivalent for aerobic oxidation and denitrification, respectively). We infer that there are fewer phosphorylation sites in the electron transport system associated with the reduction of nitrate to nitrogen gas than in that associated with aerobic respiration.

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