APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Nov. 1993, p. 3951–3953 Vol. 59, No. 11
0099-2240/93/113951-03$02.00/0

Effect of Medium Composition on the Denitrification of Nitrate by *Paracoccus denitrificans*

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Received 2 November 1992/Accepted 10 August 1993

The course of denitrification of nitrate in static cultures of *Paracoccus denitrificans* was studied. Reduction of nitrate to gaseous nitrogen without accumulation of nitrite because of parallel and balanced activities of nitrate and nitrite reductases was observed in nutrient broth. In minimal liquid cultures supplemented with either methanol, acetate, or ethanol as a sole carbon source, substantial amounts of nitrite (up to 70%) accumulated. The reduction in nitrite concentration began just after the transformation of nitrate to nitrite was completed. The addition of some growth factors to minimal media shortened the bacterial biomass doubling time. A correlation coefficient of 0.71 between the doubling time and the amount of accumulated nitrite in cultures was found. My results indicated that the type of denitrification carried out by *P. denitrificans* is not stable and depends on the nutritional composition of the culture medium.

In continuous cultures of a mixed population of denitrifying bacteria grown in medium supplemented with nitrate nitrite accumulation is often observed. The amount of accumulated nitrite depends on a number of factors. One important factor seems to be the type of organic compound used as the carbon source. During denitrification of 1,000 mg of NO_3N per liter under optimal conditions in packed bed reactors containing minimal medium supplemented with methanol, minimal medium supplemented with acetic acid, and minimal medium supplemented with ethanol, the amounts of accumulated nitrite are 150, 40, and 50 mg of N per liter, respectively (5, 8). When starch was used as a carbon source, the number of denitrifying bacteria clearly depended on the ratio of starch concentration to nitrate concentration in the medium. Therefore, starch at certain concentrations resulted in the selection of fermenting bacteria capable of reducing nitrate to only nitrite (9). The levels of accumulated nitrites have also been found to depend on the initial concentration of nitrate in the medium. At high concentrations (3 or 4 g of NO_3 N per liter of medium) the amount of accumulated nitrite was almost 1 g of N per liter in the effluent (4).

Accumulation of nitrite is also strongly affected by the species composition of the culture. Comparative studies on the denitrification of nitrate by three strains of denitrifying bacteria isolated from waste treatment devices (*Paracoccus denitrificans*, *Pseudomonas stutzeri*, and *Pseudomonas aeruginosa* [3]) revealed the existence of three patterns of nitrate denitrification. *Paracoccus denitrificans* in broth cultures does not accumulate nitrite. *Pseudomonas stutzeri* first completely transforms nitrate to nitrite, and then nitrite is denitrified to nitrogen gas. Finally, *Pseudomonas aeruginosa* denitrifies nitrate with transient accumulation of nitrite in the medium. These results strongly suggest that there are differences among bacterial species and triggered the this study on the dependence between type of denitrification and environmental conditions.

An examination of the course of nitrate denitrification by *Paracoccus denitrificans* and the dependence of this process on the composition of the culture medium was the main goal of this investigation. *Paracoccus denitrificans* was isolated from a waste treatment device during denitrification (4) and was maintained aerobically on Difco nutrient agar slants at 4°C.

Static cultures of *Paracoccus denitrificans* were grown in mineral medium supplemented with either methanol, sodium acetate, or ethanol at the same concentration (1.0 g/liter) that resulted in complete denitrification of 17.9 mmol of nitrate per liter. Mineral medium contained (per liter) 7.0 g of K_2HPO_4, 3.0 g of KH_2PO_4, 0.5 g of sodium citrate · $2H_2O$, 0.1 g of $MgSO_4 \cdot 7H_2O$, 1.0 g of $(NH_4)_2SO_4$, 0.05 g of $FeSO_4 \cdot 7H_2O$, and 1.8 g of KNO_3 (17.9 mmol/liter). A *Paracoccus denitrificans* culture grown in Difco nutrient broth enriched with KNO_3 at a concentration of 1.8 g/liter was used as a control. Bacterial cultures (100 ml) were grown statically in 100-ml cylinders sealed with rubber stoppers to ensure anaerobic conditions. Media were degassed for 30 min at 100°C prior to inoculation. All samples were removed with a syringe. A 24-h culture of *Paracoccus denitrificans* grown in petri plates was washed, suspended in a saline solution, and used as the inoculum. The density of the culture at the beginning of the experiments was 0.1. All static cultures were incubated at 30°C. The changes in optical density and in nitrate and nitrite concentrations in *Paracoccus denitrificans* cultures are shown in Fig. 1. The optical densities of bacterial cultures grown in nutrient broth and minimal media were determined at 600 and 410 nm, respectively. Nitrate and nitrite concentrations were estimated by the method of Hermanowicz et al. (6).

The greatest increases in bacterial biomass during denitrification of nitrate were observed in nutrient broth cultures. In mineral medium supplemented with ethanol the increase in bacterial biomass was comparable to the increases in cultures grown in nutrient broth. However, *Paracoccus denitrificans* grew very poorly in mineral medium supplemented with methanol or sodium acetate.

The denitrification processes in minimal and nutrient broth media differed significantly; e.g., 12 h of incubation was necessary to denitrify nitrate in cultures grown in nutrient broth, whereas as much as 6, 9, and 15 days were needed to reduce the same amounts of nitrate in mineral media supplemented with ethanol, sodium acetate, and methanol, respectively. Accumulation of nitrite was observed in all minimal media; a maximum of 12.1 mmol of nitrite per liter accumulated in cultures supplemented with methanol and sodium

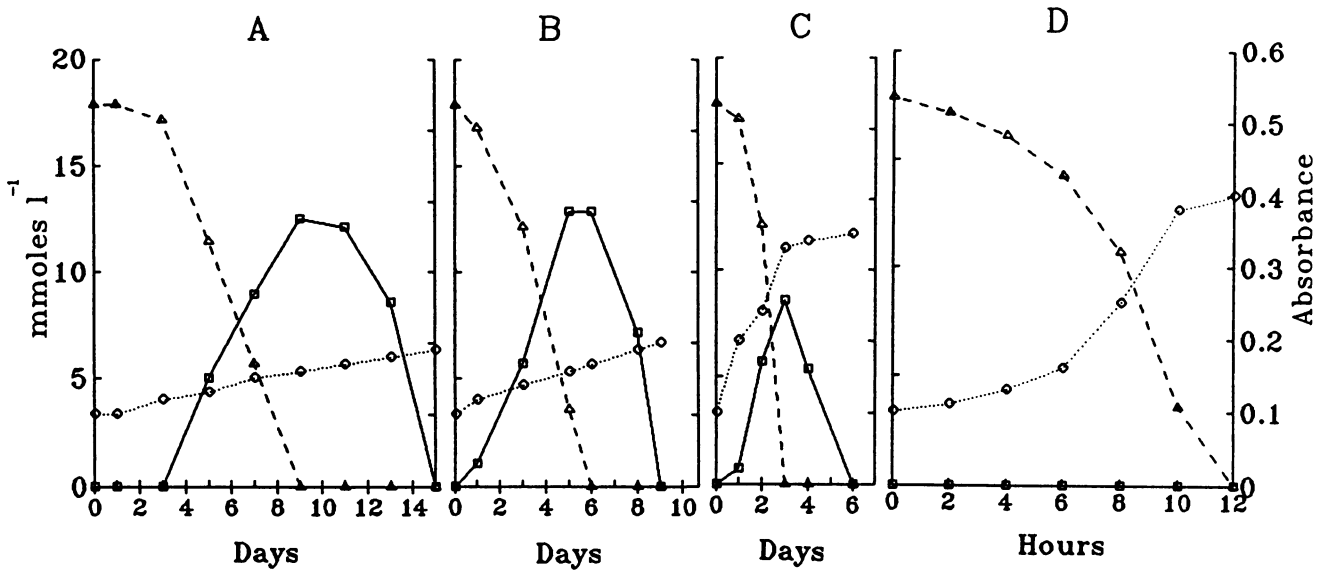

FIG. 1. Growth of *Paracoccus denitrificans* and denitrification of nitrates in static cultures grown in minimal media supplemented with methanol (A), sodium acetate (B), and ethanol (C) and in a control culture grown in nutrient broth containing nitrates (D). Symbols: ○, growth curve; □, nitrite concentrations in cultures (in millimoles per liter); △, nitrate concentrations in cultures (in millimoles per liter).

acetate, and 8.6 mmol of nitrite per liter accumulated in cultures supplemented with ethanol. The accumulated nitrite was completely reduced after 6 days of incubation with methanol and after 3 days of incubation with acetate or ethanol. The maximum level of accumulated nitrite in the control nutrient broth culture was 0.04 mmol/liter after 2 h of incubation. The results shown in Fig. 1 strongly suggest that accumulation of nitrite during nitrate reduction by *Paracoccus denitrificans* (carried out in minimal media) depends both on the energetic quantity of the organic compounds that serve as sole carbon sources and on the amount of nitrate. Despite the induction of several dissimilatory enzymes in anaerobic cultures of *Paracoccus denitrificans* (1), it seems evident that in the presence of relatively high concentrations of nitrate, nitrate reduction takes place in two steps; the reduction of nitrate to nitrite is followed by the reduction of nitrite to gaseous nitrogen. Hence, I suggest that lack of accumulation of nitrite depends on an appropriate equilibrium between the organic carbon source concentration and the nitrate concentration. This suggestion is supported by the observations that substantially less nitrite accumulated in the bacterial cultures grown in ethanol-containing minimal medium and that quite similar levels of nitrite accumulated in cultures containing methanol and cultures containing sodium acetate.

In additional experiments the level of nitrite accumulation was compared with the increase in biomass of *Paracoccus denitrificans*. Cultures were grown on mineral media that contained either methanol, sodium acetate, or ethanol as a sole carbon source, as well as the same media enriched with some supplements. These supplements were yeast extract (Difco) (0.1 g/liter) and the vitamins thiamine, cobalamine, riboflavin, pantothenic acid, pyridoxin, and nicotinic acid (Sigma) (0.1 mg/liter). Table 1 shows clearly that the lack of accumulation of nitrite detected during the denitrification of nitrate by *Paracoccus denitrificans* is not a constant feature but strongly depends on the quality of the growth medium. The growth rates of *Paracoccus denitrificans* also varied depending on the level of nutrients. The doubling times for bacterial biomass calculated from optical density values were 161 h for bacteria grown in the presence of methanol as a sole carbon source and as little as 2.1 h for the same strain grown in nutrient broth. The coefficient of correlation between the doubling time and the amount of accumulated nitrite was estimated to be 0.71. However, this coefficient of correlation applies only to bacterial doubling times between 2.1 and 52 h. When the doubling time is more than 52 h, the level of accumulation of nitrite decreases to values between 0.04 and 12.2 mmol/liter, and these values do not change even when the biomass doubling time increases.

The bacterial biomass doubling time varied significantly because of changes in the nutritional composition of the growth media. *Paracoccus denitrificans* is a prototrophic bacterium that grows relatively well in minimal media and in particular in media containing ethanol (doubling time, 36 h). In ethanol-containing minimal medium enriched with yeast

TABLE 1. Levels of the nitrite accumulated compared with medium composition in static cultures of *Paracoccus denitrificans*

Organic carbon source	Supplement(s)	Doubling time (h)[a]	% of nitrite accumulated
Methanol		161.2	70
Acetate		100.8	72
Methanol	Thiamine + cobalamine	51.8	68
Ethanol		36.6	48
Ethanol	Cobalamine	26.1	50
Methanol	Yeast extract	14.7	48
Ethanol	Thiamine	9.2	34
Acetate	Yeast extract	9.0	44
Ethanol	Yeast extract	7.5	12
Ethanol	B group vitamins	7.5	12
Ethanol	Thiamine + cobalamine	7.2	12
Broth		2.1	Tr

[a] Biomass doubling times were calculated from optical density values.

extract, group B vitamins, or just cobalamine and thiamine, the biomass doubling time decreased to 7.2 to 7.5 h, while the level of nitrite accumulation was only 2 mmol/liter. The use of methanol-containing medium supplemented with yeast extract and methanol-containing medium supplemented with cobalamine plus thiamine resulted in shortened bacterial doubling times of 14.7 and 51.7 h, respectively, while the levels of nitrite that accumulated were 8.6 and 12.2 mmol/liter, respectively. These results suggest that enrichments added to growth media had no direct influence on nitrite accumulation and, therefore, no direct effect on the induction time and efficiency of nitrite reductase.

There may be several reasons for the accumulation of nitrite in denitrifying bacterial cultures during nitrate reduction: (i) inhibition of nitrite reductase by nitrate because of competitive utilization of nitrate as an acceptor of electrons in the presence of nitrite (7); (ii) inhibition of nitric oxide reductase by nitrate which precedes the inhibition of nitrite reductase by the accumulated nitric oxide (10); (iii) unbalanced reduction reactions for nitrate and nitrite catalyzed by appropriate reductases (2); and (iv) delayed induction of nitrite reductase compared with nitrate reductase (11).

The results presented in this paper and by Blaszczyk (3) support the last possibility. In nutrient broth cultures the induction of nitrate reductase and the induction of nitrite reductase occur either simultaneously or in quick succession. However, in minimal medium, in which the synthesis of nitrate reductase takes place relatively early, the induction of nitrite reductase is delayed for several hours to several days.

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