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Comparison of Denitrification Between *Paracoccus* sp. and *Diaphorobacter* sp.

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Abstract Denitrification was compared between *Paracoccus* sp. and *Diaphorobacter* sp. in this study, both of which were isolated from activated sludge of a denitrifying reactor. Denitrification of both isolates showed contrasting patterns, where *Diaphorobacter* sp. showed accumulation of nitrite in the medium while *Paracoccus* sp. showed no accumulation. The nitrate reduction rate was 1.5 times more than the nitrite reduction in *Diaphorobacter* sp., as analyzed by the resting state denitrification kinetics. Increasing the nitrate concentration in the medium increased the nitrite accumulation in *Diaphorobacter* sp., but not in *Paracoccus* sp., indicating a branched electron transfer during denitrification. *Diaphorobacter* sp. was unable to denitrify efficiently at high nitrate concentrations from 1 M, but *Paracoccus* sp. could denitrify even up to 2 M nitrate. *Paracoccus* sp. was found to be an efficient denitrifier with insignificant amounts of nitrite accumulation, and it could also denitrify high amounts of nitrate up to 2 M. Efficient denitrification without accumulation of intermediates like nitrite is desirable in the removal of high nitrates from wastewaters. *Paracoccus* sp. is shown to suffice this demand and could be a potential organism to remove high nitrates effectively.

Keywords Denitrification · Wastewater treatment · *Paracoccus* sp. · *Diaphorobacter* sp. · High nitrate removal

Introduction

Denitrification is the bacterial respiratory process that couples electron transport phosphorylation with sequential reduction of nitrate to nitrogen through the intermediates nitrite, nitric oxide, and nitrous oxide [34]. The denitrifying ability of bacteria is widely distributed among several genera, though the frequency is more among the alpha and beta proteobacteria [35]. However, the reduction rate of nitrogenous oxides during the

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denitrification process varies in different species and even strains [4, 5]. Environmental factors are known to significantly affect the denitrification process [12, 28, 29]. Partial pressure of oxygen is reported to determine the synthesis of reductases differentially, and nitrate is also known to affect nitrite reduction in *Pseudomonas stutzeri* [17]. Almeida et al. [2] observed the dependency of nitrite reduction on the nitrate concentrations in *Pseudomonas flourescens*. Low nitrite than nitrate reduction causes the buildup of nitrite, and the accumulated nitrite limits denitrification and growth [1]. Almeida et al. [2] showed that nitrite concentrations above 130 mg NL^{-1} limit the growth of *P. flourescens*.

Wastewaters of certain industries producing chemicals, fertilizers, explosives, etc., contain very high amounts of nitrate with more than 1,000 mg L^{-1} [7, 10, 13, 33], which, when released into the environment such as lakes or rivers, causes eutrophication and also contaminates the drinking water. The WHO guideline values for nitrate and nitrite in drinking water are 50 and 3 mg L^{-1}, respectively [31], because consumption of high nitrate and nitrite concentrations are known to cause methemoglobinemia in infants and other health hazards. Biological denitrification is the widely used phenomenon for nitrate removal from wastewaters. However, prominent limiting conditions for efficient denitrification to occur in reactors are high nitrate concentrations in wastewaters [6, 8, 11] and the accumulation of nitrite [12, 26]. Francis and Mankin [11] observed nitrate concentrations above 6 kg L^{-1} to inhibit nitrate reduction. High nitrate concentrations possibly limit denitrification because of their chaotropic effect and also because the denitrification intermediates, nitrite and nitric oxide, generate reactive nitrogen species (RNS) which are harmful to cells [25].

Thus, denitrifiers which can reduce high nitrate concentration without accumulation of intermediates are a necessity in denitrifying reactors to increase the process efficiency. In order to check the denitrification potential of the bacteria present in denitrifying reactors, we isolated two cultures and compared their denitrification patterns at different nitrate concentrations. The cultures under study were *Paracoccus* sp. W1b (henceforth *Paracoccus*) and *Diaphorobacter* sp. D1 (henceforth *Diaphorobacter*), both isolated from activated sludge of a denitrifying reactor. Different strains of both cultures are known to thrive in sludge habitats [15, 20, 21, 32]. Our study shows that *Paracoccus* could denitrify more efficiently and also tolerate high nitrate concentrations than *Diaphorobacter*.

Materials and Methods

Isolation and Identification of Bacteria

Isolates W1b and D1 were isolated from the denitrifying reactor sludge of a fertilizer factory on peptone nitrate agar plates. Identification of the isolates was done by sequencing the partial 16S rRNA gene. The primers used for amplifying 16S rRNA gene by PCR were 27f (5′-GAGAGTTTGATCTGGCTCAG-3′) and 1541r (5′-AAGGAGGTGATCCAGCCG-3′). Sequencing of the PCR amplicon was done in ABI *3730xl* DNA Analyzer at services provided by XcelrisLabs. The primers used for sequencing the PCR product were 27f, 1541r, and also 341f (5′-CTACGGGAGGCAGCA-3′), 534r (5′-ATTACCGCGGCTGCTGG-3′), and 1107r (5′-GCTCGTTGCGGGACTTAA-3′) according to Pillai and Archana [23]. Overlapping sequences were analyzed in Mega 4.1 software [19] and corrected to obtain a larger fragment of the 16S rRNA gene. The sequences are submitted to the NCBI data bank with accession numbers HQ625227 and HQ625228.

Culture Conditions

Culture maintenance and inoculum preparation was done in peptone nitrate medium (PNB). Denitrification experiments were performed in MM2 medium [27] consisting of sodium succinate 7.9 g, $MgSO_4 \cdot 7H_2O$ 0.2 g, K_2HPO_4 0.2 g, $FeSO_4 \cdot 7H_2O$ 0.05 g, $CaCl_2 \cdot 2H_2O$ 0.02 g, $MnCl_2 \cdot 4H_2O$ 0.002 g, $NaMoO_4 \cdot 2H_2O$ 0.001 g, KNO_3 1.0 g, yeast extract 1.0 g, pH 7.0, and distilled water 1,000 mL. A 24-h-old culture was centrifuged, washed, and resuspended in phosphate-buffered saline (PBS) for inoculation in denitrification experiments. Incubation of the cultures was done at 30 °C in static conditions for maintenance of anoxia.

Denitrification Studies

Denitrification during growth was monitored up to 36 h with nitrate and nitrite measured in intervals of 6 h. The experiment was performed in 250-mL Erlenmeyer flasks in MM2 medium containing 10 mM KNO_3 inoculated with 10^8 cells mL^{-1} of a 24-h-old culture.

Nitrate reduction, nitrite formation, and the relative rates (RR) of nitrate and nitrite reduction were calculated according to Dhamole et al. [9], where relative rate is described as

$$\mathrm{RR} = \frac{K_{\mathrm{NO3}}}{K_{\mathrm{NO3}} - k_{\mathrm{NO2}}}$$

where K_{NO3} is the rate of nitrate reduction and k_{NO2} is the rate of nitrite formation in the presence of nitrate.

Nitrate and nitrite reduction rates by resting cell suspension were performed as follows: Cells grown for 24 h in peptone nitrate broth were harvested by centrifugation at 10,000 rpm for 5 min, washed twice with phosphate-buffered saline (pH 7.4), and resuspended in PBS. Succinate and nitrate were added as electron donor and acceptor, respectively, and the reductions of nitrate and nitrite were estimated for 20 min.

Denitrification experiments at high nitrates were done in 24-well microtiter plates using 3 mL MM2 medium with the C/N ratio of 5.0 maintained at appropriate nitrate concentration. High nitrate concentrations were used in this experiment from 0.1 to 2 M. About 10^8 cells mL^{-1} were used as inoculum and incubated for 12 h, after which colony-forming units (CFU), nitrate, and nitrite were measured.

Analytical Methods

Nitrate was estimated according to the method described by Jenkins and Medsker [14]. Nitrite was determined according to the standard method described in APHA [3]. The method of Lowry et al. [22] was used for protein estimation, with bovine serum albumin as a standard.

Production of nitrous oxide by the culture was analyzed by withdrawing the sample from the headspace of the test tube in which the isolate was grown for 18 h in PNB medium, and N_2O was measured by gas chromatography (Perkin Elmer, Auto system XL) with an electron capture detector.

Results

Characterization of the Isolates

The two cultures under study, W1b and D1, were isolated from the denitrifying reactor sludge sample of a fertilizer industry in peptone nitrate medium. The presence of nitrous oxide in the headspace of the growth tube confirmed them to be denitrifiers. Isolate W1b was observed to be a Gram-negative cocci, and BLAST results of 1,094-bp partial 16S rRNA gene showed 99% identity with *Paracoccus* species; isolate D1, a Gram-negative rod, showed 98% similarity with *Diaphorobacter* species with a 1,437-bp partial 16S rRNA gene sequence. Phylogenetic positions of both the isolates are shown in Fig. 1, where the isolates W1b and D1 clustered with *Paracoccus* sp. and *Diaphorobacter* sp., respectively.

Denitrification Pattern of the Isolates

The nitrate reduction and nitrite accumulation of both isolates were monitored in batch mode up to 36 h in 250-mL Erlenmeyer flasks under static condition. Nitrate was reduced to 1.65 mM from 12.0 mM in 36 h by *Paracoccus*, with insignificant amount of nitrite accumulation at the 6-h interval (Fig. 2). *Diaphorobacter* could reduce nitrate from 12.0 to

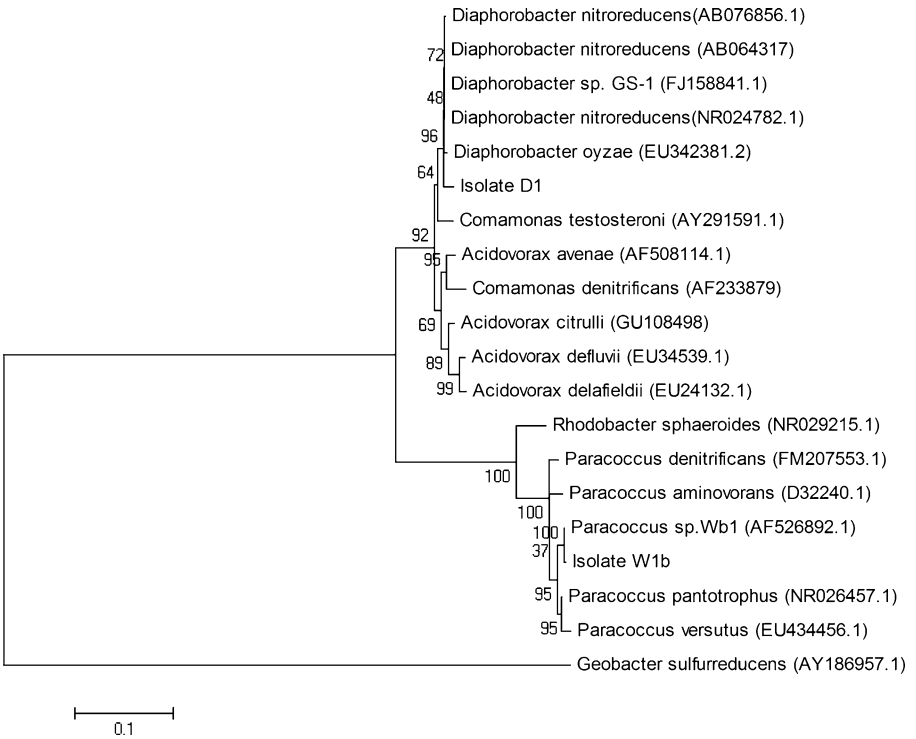

Fig. 1 Phylogenetic tree constructed by neighbor-joining method showing position of the isolates with other related cultures. Bootstrap analysis of 1,000 resampling by maximum-likelihood method was used to construct tree. *Parenthesis* contains the accession number of the cultures

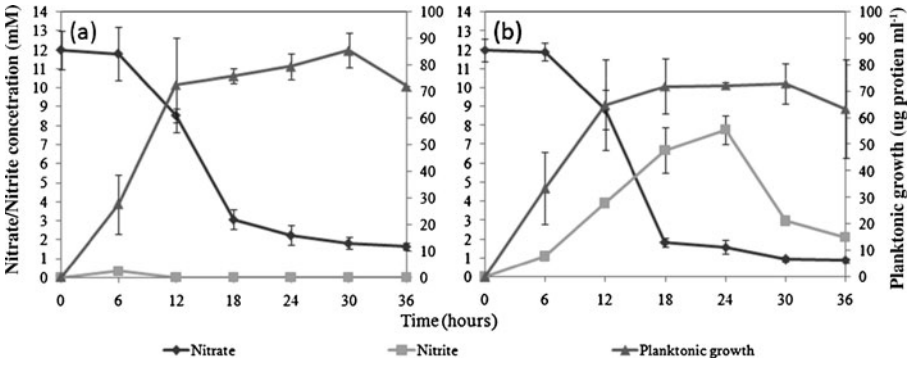

Fig. 2 Denitrification pattern of the isolates (**a**) *Paracoccus* and (**b**) *Diaphorobacter*. *Error bars* represent the standard deviation

0.89 mM in 36 h, whereas a substantial amount of nitrite was accumulated in the medium (Fig. 2). Accumulation of nitrite increased to around 7.8 mM in 24 h and decreased to 2.1 mM at 36 h. The calculated RR was high in *Diaphorobacter* and nearly 1.14 in *Paracoccus* (Table 1), suggesting that the rate differences between nitrate and nitrite reduction is the possible reason for nitrite accumulation.

The reduction rates of nitrogenous oxides were further estimated in the resting state of these cultures. The nitrate reduction rate was 1.5-fold higher than the nitrite reduction rate in *Diaphorobacter*, whereas the nitrate to nitrite reduction rate ratio of *Paracoccus* was found to be 1.0 (Table 2), suggesting similar reduction rates of nitrate and nitrite. However, the influence of increasing nitrate concentrations on the reduction of nitrite showed an increased accumulation of nitrite in *Diaphorobacter*, whereas nitrite buildup was not observed in *Paracoccus* with the increase in nitrate concentration (Fig. 3). The nitrate reduction rate at increasing nitrate concentrations is shown in Table 3, where the nitrate reduction rate increased substantially with 10 mM nitrate.

Influence of C/N Ratio on Denitrification by the Isolates

The amount of carbon source plays a crucial role in reducing nitrates in denitrifying reactors, so the optimum C/N ratio was tested for efficient denitrification in the cultures. Nitrate was provided as both electron acceptor and nitrogen source in the medium to the bacteria. Growth and nitrate reductions were significantly affected in both the isolates at a C/N ratio of <1, but much differences were not found at increasing C/N ratios more than 1.0 (Fig. 4a, d). An increase in nitrite accumulation was seen at decreasing C/N ratio in

Table 1 Calculated rates during denitrification deduced from data shown in Fig. 2

Isolates	NO_3 reduction ($\mu M\ min^{-1}$)	NO_2 formation ($\mu M\ min^{-1}$)	RR
Paracoccus sp.	7.22	0.9	1.14
Diaphorobacter sp.	9.72	6.1	2.68

Rates were calculated according to Dhamole et al. [9]. RR = 1 implies no nitrite buildup, whereas RR > 1 signifies nitrite accumulation

RR relative rate of nitrate and nitrite reduction

Rates determined by linear regression. $R^2 > 0.87$

Table 2 Nitrate and nitrite reduction rates by resting-state cells

Isolates	NO_3 reduced ($\mu M\ mg^{-1}$ protein min^{-1})	NO_2 reduced ($\mu M\ mg^{-1}$ protein min^{-1})	Ratio of nitrate to nitrite reduction
Paracoccus sp.	159.0±85.0	147.0±15.0	1.08
Diaphorobacter sp.	117.0±13.0	78.0±14.0	1.50

Paracoccus, though significant nitrite accumulation changes were not observed in *Diaphorobacter* with changes in the C/N ratio (Fig. 4b, e). A 27-fold increase in nitrite was seen in a C/N ratio of 0.25 than 1.0, and a C/N ratio of 0.5 showed a 9-fold increase in nitrite than the C/N ratio of 1.0 in *Paracoccus* (Fig. 4b). Nitrite accumulation was in the range of 3.5–6.9 mM in *Diaphorobacter* at all C/N ratios (Fig. 4e).

High Nitrate Denitrification by the Isolates

Nitrate reduction and accumulation of nitrite was investigated in both the isolates at high nitrate concentrations, the results of which are shown in Table 4. A C/N ratio of 5.0 was maintained at all nitrate concentrations tested. *Paracoccus* reduced nitrate in the range 54–76.5% in 12 h for all the nitrate concentrations tested. Nitrite accumulation was in the range 1.15–3.4 mM, although 6.5 mM nitrite accumulated with 0.5 M initial nitrate. An average CFU of $10^9\ mL^{-1}$ was maintained at all tested nitrate concentrations (Table 4). Nitrate reduction was high in *Diaphorobacter* in the range 70–80% up to 0.5 M initial nitrate concentration, whereas a drastic decrease in nitrate reduction with 17.5% and 15.7% and a 2 log decrease in CFU with 10^7 cells were observed when nitrate was increased to 1 and 2 M, respectively. Nitrite accumulation in *Diaphorobacter* was higher than *Paracoccus*, with a range of 8.1–13.6 mM.

Discussion

Though similar denitrification apparatus is present in diverse groups of bacteria, the denitrifying activity of each step differs in different organisms. In this study, we compared

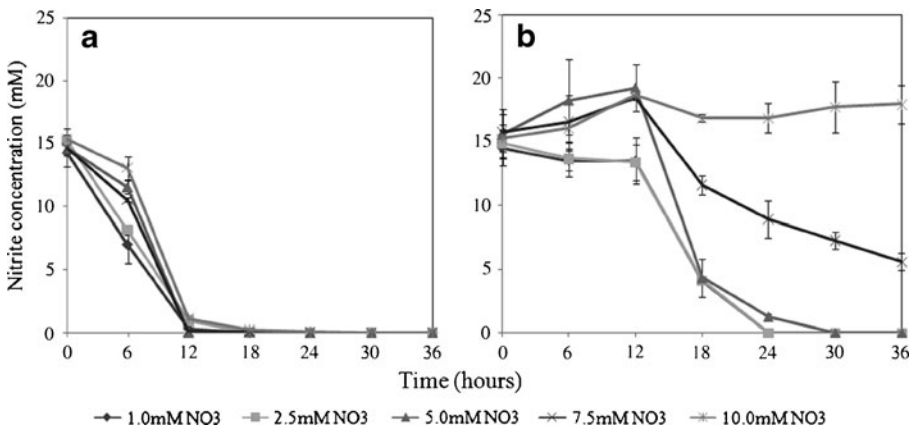

Fig. 3 Nitrite accumulation at increasing nitrate concentrations in the medium. **a** *Paracoccus*, **b** *Diaphorobacter*. *Error bars* represent the standard deviation

Table 3 Nitrate reduction rates during denitrification at increasing nitrate concentrations

Initial nitrate concentration (mM)	Nitrate reduction (μM min^{-1})	
	Paracoccus	*Diaphorobacter*
1.0	3.08	5.25
2.5	4.16	5.00
5.0	4.16	4.16
7.5	5.27	5.27
10.0	8.88	9.44

Rates determined by linear regression. $R^2 > 0.87$

the denitrification pattern of two cultures, W1b and D1, isolated from the activated sludge of a denitrifying reactor and identified as *Paracoccus* and *Diaphorobacter*, respectively (Fig. 1). The nitrate reduction rate is higher in *Diaphorobacter*, but *Paracoccus* reduced nitrate without much accumulation of nitrite transiently, unlike *Diaphorobacter* which accumulated nitrite significantly (Fig. 2). *Diaphorobacter* species are reported to also nitrify ammonia to nitrite [16]; however, ammonia was not provided in the MM2 medium (refer to "Materials and Methods" for composition), suggesting that the nitrite accumulation was due to nitrate reduction.

Accumulation of nitrogenous oxide intermediates during denitrification was explained by Betlach and Tiedje [4] where they showed that nitrite accumulation in *Alcaligenes* sp. and *P. flourescens* was due to the differences in the reduction rates of nitrate and nitrite. Investigating nitrate and nitrite reduction rates in resting cell suspensions provides insight into the overall reduction rate, including the flux of nitrate/nitrite into and outside the cell. It was observed that *Paracoccus* have a nitrate-to-nitrite reduction ratio of 1.08, suggesting nearly equal rates in the reduction of these two nitrogenous oxides, but *Diaphorobacter* showed a ratio of 1.5 (Table 2). High nitrate than nitrite reduction rate was the possible reason for the accumulation of nitrite in *Diaphorobacter*. Increasing nitrate concentrations

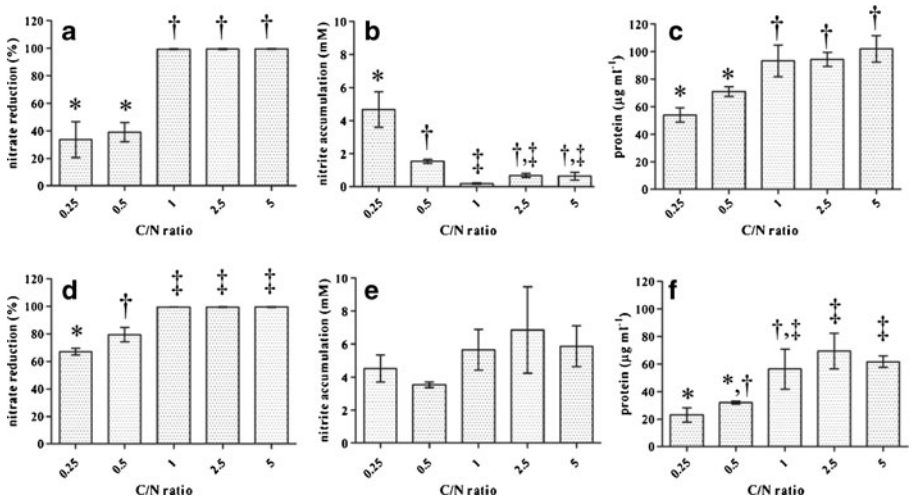

Fig. 4 Influence of the C/N ratio on nitrate reduction, nitrite accumulation, and growth of *Paracoccus* (**a**–**c**) and *Diaphorobacter* (**d**–**f**). *Bars* represent the average values of at least three independent experiments. *Error bars* represent the standard deviation. *Same symbols above the bars* indicate no significant difference. One-way ANOVA with Tukey test was used to determine significant differences ($p<0.05$)

Table 4 Denitrification and growth of the isolates at high nitrate concentrations

Nitrate concentration (M)					Nitrite accumulation (mM)		CFU[a] ($\times 10^8$)	
Initial	*Paracoccus*		*Diaphorobacter*					
	Final	% reduced	Final	% reduced	*Paracoccus*	*Diaphorobacter*	*Paracoccus*	*Diaphorobacter*
0.1	0.046±0.004	54.0	0.029±0.002	71.0	1.55±0.40	11.3±1.55	10.0	30.0
0.2	0.047±0.0005	76.5	0.040±0.003	80.0	1.15±0.15	13.6±1.45	10.0	50.0
0.5	0.198±0.003	60.4	0.150±0.002	70.0	6.50±2.15	11.8±0.85	10.0	27.0
1.0	0.404±0.077	59.6	0.825±0.246	17.5	2.00±0.45	8.1±4.1	15.0	0.30
2.0	0.693±0.127	65.3	1.686±0.332	15.7	3.40±0.20	9.05±2.05	13.5	0.56

[a] Initial CFU=10^8

in the medium increased the nitrite buildup in *Diaphorobacter*, whereas nitrite accumulation was not observed in *Paracoccus* (Fig. 3). The lower nitrite than nitrate reduction could be the possible reason for the nitrite buildup in *Diaphorobacter*, but a ratio of 1.08 (Table 2) for nitrate to nitrite reduction found in *Paracoccus* could not explain the increased reduction rate of nitrite when nitrate concentrations were increased in the medium. Branched electron flow to the nitrogenous oxides in *Paracoccus denitrificans* has been reported by Kucera et al. [18]. Similarly, the increased nitrite reduction with the increase in nitrate concentration observed for *Paracoccus* (Fig. 3) suggests "inhibition by product via respiratory chain" [18] to be the possible phenomenon.

The C/N ratio significantly influenced nitrate reduction and growth in both the isolates (Fig. 4). Low C/N ratio also affected nitrite accumulation significantly in *Paracoccus*. Carbon source acts as an electron donor; hence, higher amounts of carbon source than nitrate is required to completely reduce nitrates. A C/N ratio of 5.0 was provided in further studies of high nitrate reduction. High nitrate concentrations tested did not affect the nitrate reduction and growth significantly in *Paracoccus*, but a substantial drop in nitrate reduction and growth was observed in *Diaphorobacter* from 1.0 M nitrate concentration (Table 4). Nitrates in excess can be harmful to the cell because of their chaotropic effect. A *Klebsiella oxytoca* strain was isolated by Pinar et al. [24], which could tolerate nitrate up to 1.0 M, but *Klebsiella* species are also reported to have the property of dissimilatory nitrate reduction to ammonium [30]. However, *Paracoccus* could denitrify efficiently even at 2.0 M nitrate concentration and tolerate up to 4.0 M nitrate concentrations (data not shown). The denitrification intermediates, nitrite and nitric oxide, generate RNS which are more toxic to the cells [25]. Efficient denitrification by branched electron transfer [18] in *Paracoccus* is possibly the mechanism to detoxify its microenvironment, whereas the accumulation of nitrite in *Diaphorobacter* possibly lowered the fitness of the cell at high nitrate concentrations.

The above results suggest that *Diaphorobacter* possibly transfers electron sequentially in the denitrification system from nitrate to dinitrogen formation, whereas a branched electron transfer occurs in *Paracoccus*. The branched electron transfer strategy of *Paracoccus* might help the organism adapt to environments containing high nitrogenous oxide concentrations like industrial wastewaters. We have also reported studies on biofilm formation by *Paracoccus* elsewhere [27], and our unpublished data on biofilm community analysis by fluorescence in situ hybridization probes show an increase of *Paracoccus* sp. in the biofilm community at nitrate concentrations above 250 mM in a 1-L laboratory reactor. Efficient denitrification complemented with surface colonization by biofilm formation gives this isolate potential in high nitrate removal processes in denitrifying reactors.

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