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Reduction of Oxidized Inorganic Nitrogen Compounds by a New Strain of *Thiobacillus denitrificans*

JACQUES BALDENSPERGER and JEAN-LOUIS GARCIA
Laboratory of Soil Microbiology, ORSTOM, Dakar, Sénégal

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Abstract. Denitrification by *Thiobacillus denitrificans* "RT" strain was investigated using manometry and gas chromatography.

1. From nitrate, resting cells produced only nitrogen anaerobically with thiosulfate as the electron donor. The data suggest that nitrate was assimilated and dissimilated by the same nitrate reductase, assayed with benzyl-viologen as the electron donor.

2. From nitrite, whole cells produced nitric oxide, nitrous oxide and nitrogen, using thiosulfate as the electron donor; nitrogen was the final product of the reduction. Crude extract reduced nitrite to nitrogen with p-phenylene-diamine and dimethyl-p-phenylene diamine as the electron donors, and produced nitric oxide, nitrous oxide and nitrogen with tetramethyl-p-phenylene-diamine as the electron donor. Nitrite was reduced to nitric oxide and nitrous oxide by crude extract using ascorbate-phenazine methosulfate as the electron donor.

3. From nitric oxide, whole cells produced nitrous oxide and nitrogen using thiosulfate as the electron donor, nitrogen was the final reduction product. Nitric oxide was reduced to nitrous oxide by crude extract with the ascorbate-phenazine methosulfate system.

4. Whole cells reduced nitrous oxide to nitrogen with thiosulfate as the electron donor.

It was not possible to detect any nitrous oxide reductase activity in crude extract.

5. A scheme was formulated as a possible mechanism of denitrification by *Thiobacillus denitrificans* "RT" strain.

Key words: Thiobacillus denitrificans — Reduction of Nitrate — Nitrite — Nitric Oxide — Nitrous Oxide.

A new strain of denitrifying chemolithotrophic sulfo-oxidizer, *Thiobacillus denitrificans* "RT" strain, was isolated from a rice soil in the estuary of the river Senegal, using a thiosulfate anaerobic medium with nitrate as the sole nitrogen source.

The purpose of this paper is to report investigations of the respiratory nitrate reduction chain of the organism.

Materials and Methods

Organism. Thiobacillus denitrificans "RT" was isolated by enrichment cultures in the anaerobic liquid medium of Baalsrud and Baalsrud (1954) devoid of ammonium nitrogen. Except for its ability to use nitrate as sole nitrogen source, our isolate had similar morphological and physiological characteristics with *Thiobacillus denitrificans* "Oslo" strain from K. S. Baalsrud.

Media. Stock cultures of *Thiobacillus denitrificans* "RT" were maintained anaerobically on agar plates. The solid medium contained (g/l): $Na_2S_2O_3 \cdot 5\,H_2O$, 5.0; KH_2PO_4, 1.2; K_2HPO_4, 2.0; KNO_3, 2.0; $MgSO_4 \cdot 7\,H_2O$, 0.6; Special Agar Noble (DIFCO Laboratories), 16.0; trace metal solution, 1.0 ml.

All components except K_2HPO_4 and the trace metal solution were sterilized 20 min at 120° C in 800 ml of distilled water. The dipotassium hydrogen phosphate and the trace metal solution, each dissolved in 100 ml of distilled water, were sterilized separately by filtration and added before solidification.

The trace metal solution had the following composition (g/l): MgO, 10.75; $FeSO_4 \cdot 7\,H_2O$, 38.0; $CaCO_3$, 2.0; $ZnSO_4 \cdot 7\,H_2O$, 1.44; $MnSO_4 \cdot 4\,H_2O$, 1.12; $CuSO_4 \cdot 5\,H_2O$, 0.25; $CoCl_2 \cdot 5\,H_2O$, 0.25; $NiCl_2 \cdot 6\,H_2O$, 0.25; Bo_3H_3, 0.66; concentrated HCl, 51.3 ml.

Carbon dioxide was provided as carbon source in culture jars by "Gaspak" disposable gas generator envelopes (BBL, Cockeysville, Maryland, U.S.A.).

Isolated colonies were subcultured every 3 weeks, and if stored at 5° C remained viable for at least 4 months.

Anaerobic cultures were grown in liquid medium "DB" containing (g/l): $Na_2S_2O_3 \cdot 5\,H_2O$, 5.0; KH_2PO_4, 2.0; KNO_3, 2.0; $MgSO_4 \cdot 7\,H_2O$, 0.6; $NaHCO_3$, 1.5; trace metal solution, 1.0 ml. The first four materials were sterilized (20 min, 120° C) together in 800 ml of distilled water, while bicarbonate and trace metal solution, each dissolved in 100 ml of distilled water, were sterilized separately by filtration and added after cooling.

Anaerobic growth was conducted at 33° C under nitrogen, and the cells were harvested in a "Sharples" continuous

Non Standard Abbreviations. PDA = p-phenylene-diamine; DMPDA = N:N-dimethyl-p-phenylene-diamine; TMPDA = N:N:N′:N′-tetramethyl-p-phenylene-diamine; PMS = phenazine methosulfate; BV = benzyl-viologen.

centrifuge, washed three times at 4° C in 0.03 M phosphate buffer pH 6.8, and stored frozen prior to further use. Cell yield was about 200 mg (dry weight) per liter.

Aerobic growth was conducted either with ammonium (medium "A") or with nitrate (medium "B") as nitrogen source.

Medium "A" contained (g/l): $Na_2S_2O_3 \cdot 5\,H_2O$, 5.0; KH_2PO_4, 1.2; K_2HPO_4, 2.0; NH_4Cl, 1.0; $MgSO_4 \cdot 7\,H_2O$, 0.6; trace metal solution, 1.0 ml.

Medium "B" had the same composition except for NH_4Cl replaced by KNO_3 at the final concentration of 1 g/l.

All materials except dipotassium hydrogen phosphate and trace metal solution were sterilized 20 min 120° C in 800 ml of distilled water. Trace metal solution and K_2HPO_4, each dissolved in 100 ml of distilled water, were sterilized by filtration and added separately to the bulk of medium after cooling. The aerobic cultures were maintained at pH 6.8 by titrating sterile 10% (w/v) Na_2CO_3 into the fermentor by means of a pH stat unit. Cells were harvested and washed as for anaerobic cultures, with similar cell yield.

Preparation of Cell-Free Extracts. The washed pellets were resuspended in 0.03 M phosphate buffer pH 6.8 and passed twice through a French pressure cell at 20000 PSI. A few crystals of Deoxyribonuclease were added to the preparation which was left for 10 min at room temperature before centrifugation at 36000 × g for 90 min. The supernatant was used as "crude extract" for enzymatic studies. Protein concentration was about 35 mg/ml, and crude extracts could be stored at −20° C for at least 6 months without significant loss of activity.

Hydrogenase solution was prepared from cells of *Desulfovibrio desulfuricans* "Hildenborough" strain grown in Postgate's medium C (Postgate, 1966). After three successive washings in 0.02 M phosphate buffer pH 6, 35 g (wet weight) of cells were resuspended in 18 ml of 0.05 M phosphate buffer pH 7.6. The cell paste was stirred 10 min under nitrogen at 4° C and centrifuged 90 min at 36000 × g. The clear supernatant was used as hydrogenase fraction in the reducing mixture for manometric measurements of nitrate reduction by crude extracts of *Thiobacillus denitrificans* "RT".

Determination of Growth Rates. Growth rates were calculated in aerated conditions using a Bio-Log II biophotometer (Bonet, Maury, Jouan) and in anaerobic conditions by measuring periodically the optical density at 450 nm of 1 ml samples from 1 l cultures.

Reduction of Oxidized Inorganic Nitrogen Compounds by Washed Cells Suspensions. Reduction of nitrate, nitrite, nitric oxide and nitrous oxide by washed cells was assayed manometrically with thiosulfate as the electron donor using a model V 166 Warburg apparatus, and gas determinations were performed with a Varian Aerograph model 90 P4 gas chromatograph as previously described (Garcia, 1974).

Enzyme Assays. Nitrate reductase activity was measured in crude extracts manometrically with BV as the electron donor (Pichinoty and Piéchaud, 1968). Nitrite reductase was assayed using PDA, DMPDA, TMPDA and the PMS-ascorbate system as electron donors (Miyata and Mori, 1968; Miyata *et al.*, 1969; Pichinoty, 1969b; Iwasaki and Matsubara, 1972). Reduction of nitric oxide was measured with PMS-ascorbate (Miyata *et al.*, 1969). Nitrous oxide reductase was assayed with PDA, DMPDA, TMPDA, PMS-ascorbate, as electron donors, and with NADH, FAD and FMN according to Payne *et al.* (1971). Gas determinations were performed as for cell suspensions. *Nitrogen content* of washed cell suspension was determined by micro-Kjeldahl. *Protein* was measured by the method of Lowry *et al.* (1951) using bovine serum albumine as the reference protein.

Results

Growth Rates in Different Growth Conditions. The same generation time of 5 hrs was measured in aerated conditions of growth with ammonium (medium A) or with nitrate (medium B) as the nitrogen source, and in anaerobic cultures on nitrate (medium DB).

Nitrate Reduction by Whole Cells. Washed cell suspensions of anaerobically grown *Thiobacillus denitrificans* "RT" reduced nitrate with thiosulfate as the electron donor, with a QN_2 of 183 $\mu l N_2$/mg total microbial nitrogen/hr, and only nitrogen could be detected during the reaction.

If grown in aerobic conditions with either nitrate or ammonium as the nitrogen source, washed cells incubated anaerobically with nitrate did not evolve nitrogen with thiosulfate as the electron donor.

Nitrate Reductase in Crude Extracts. Nitrate reductase was assayed in crude extracts with the hydrogen—hydrogenase—BV system as the electron donor, as described by Pichinoty and Piéchaud (1968).

In all growth conditions, nitrate reductase and chlorate reductase activities of crude extracts were not additive; and 95% inhibition of chlorate activity was observed with 1 mM sodium azide (see Table 1).

Crude extracts from cells grown with nitrate as the sole nitrogen source in anaerobic conditions (medium DB) had a specific nitrate reductase activity of 127 μmoles NO_3^- reduced/mg protein/hr.

This activity was much lower in crude extracts from aerobically grown cells on nitrate (medium B) or ammonium (medium A) (see Table 2).

When crude extracts were centrifuged for 2 hrs at 140000 × g, about 95% of the nitrate reductase was recovered in the pellet fraction, and the specific activity of the particles was increased to 330 μmoles NO_3^- reduced/mg protein/hr.

Nitrite Reduction by Whole Cells. Washed cell suspensions from growth in anaerobic conditions with nitrate (medium DB) reduced nitrite with thiosulfate as the electron donor, and evolution of a mixture of NO, N_2O and N_2 was detected by gas chromatography. During the first 2 hrs of reaction, evolution rates of 12 μlNO, 10 $\mu l N_2O$ and 2.6 μl N_2/mg N/hr were measured.

After 12 hrs of incubation of washed cell suspensions with nitrite and thiosulfate, only nitrogen could be detected in the reaction flasks.

Table 1. Rates of hydrogen uptake by crude extracts of *Thiobacillus denitrificans* "RT"

Growth conditions		H_2 uptake (µl/hr) with addition of			
		NO_3^-	ClO_3^-	$NO_3^-+ClO_3^-$	$ClO_3^-+NaN_3^-$
NO_3^- as the terminal electron acceptor	NO_3^- as the nitrogen source [a]	570	780	540	12
O_2 as the terminal electron acceptor	NO_3^- as the nitrogen source [b]	216	288	243	30
	NH_4^+ as the nitrogen source [c]	234	360	276	18

Manometric measurements at 37° C under hydrogen, phosphate buffer pH 7, 350 µmoles; hydrogenase, 0.2 ml; BV, 500 µg; KNO_3, 50 µmoles; $KClO_3$, 50 µmoles; NaN_3, 3 µmoles; final volume 3 ml.
[a] 0.05 mg protein. [b] 8 mg protein. [c] 13 mg protein.

Table 2. Specific nitrate-reductase and chlorate-reductase activities of cell-free extracts from *Thiobacillus denitrificans* "RT"

	NO_3^- as the terminal electron acceptor	O_2 as the terminal electron acceptor	
	NO_3^- as the nitrogen source	NO_3^- as the nitrogen source	NH_4^+ as the nitrogen source
V (NO_3^-) µmole/mg prot./hr	127	0.3	0.2
V (ClO_3^-) µmole/mg prot./hr	232	0.5	0.4
V (ClO_3^-)/V (NO_3^-)	1.8	1.7	2.0

Experimental conditions as for Table 1.

Nitrite Reduction by Crude Extract. Reduction of nitrite by extracts from anaerobically grown cells of *Thiobacillus denitrificans* "RT" was assayed using PDA, DMPDA, TMPDA and the PMS—ascorbate system as electron donors.

After thermal equilibration, the side arm containing the nitrite solution was tipped into the vessel, and the chemical reaction allowed during 30 min before the enzyme fraction was tipped from the second side arm. Maximum gas evolution was observed with DMPDA as the electron donor (see Fig. 1). Gas chromatographic analysis of the atmospheres of the reaction flasks showed that only nitrogen was evolved from nitrite reduction by crude extract with PDA and DMPDA, but that a mixture of 80% NO, 4% N_2O and 16% N_2 was produced with TMPDA as the electron donor.

When nitrite reduction was assayed using the PMS—ascorbate system as the electron donor, gas chromatography showed that NO and N_2O were produced, but as simultaneous reduction of nitric oxide occurred, nitrous oxide was the final product of the reaction (Fig. 2A).

Reduction of Nitric Oxide by Whole Cells. A 10% NO concentration was used in the flask atmosphere for gas chromatographic measurements of NO reduction by whole cells with thiosulfate as the electron donor. During the first 3 hrs, a NO reduction rate of 94 µlNO reduced/mg N/hr was measured, and production of N_2O and N_2 was detected. Nitrogen was the final product of nitric oxide reduction by whole cells.

Reduction of Nitric Oxide by Crude Extract. Nitric oxide reduction by crude extract was assayed by gas chromatography, using the PMS—ascorbate system as the electron donor.

When a 10% nitric oxide concentration was used in the reaction flasks, a maximum activity of 146 µl NO reduced/mg prot/hr was measured (see Fig. 2B).

Nitric oxide was not reduced enzymatically by crude extracts of *Thiobacillus denitrificans* "RT" with PDA, DMPDA, TMPDA or NADH as electron donors.

A chemical production of N_2 with traces of N_2O was detectable by gas chromatography between NO and PDA or DMPDA, a slight reaction occurred between NO and TMPDA.

Reduction of Nitrous Oxide by Whole Cells. Washed cell suspensions from growth in anaerobic conditions in medium DB reduced nitrous oxide to nitrogen with thiosulfate as the electron donor, at the rate of 61 µl N_2O reduced/mg total microbial N/hr.

Reduction of Nitrous Oxide by Crude Extracts. It was not possible to detect any nitrous oxide reductase activity in crude extracts of *Thiobacillus denitrificans* "RT" either with PDA, DMPDA, TMPDA, PMS-ascorbate as electron donors, or with NADH, FAD and FMN as described by Payne *et al.* (1971).

Discussion

Our isolate, *Thiobacillus denitrificans* "RT", was characterized by its ability to assimilate nitrate in both

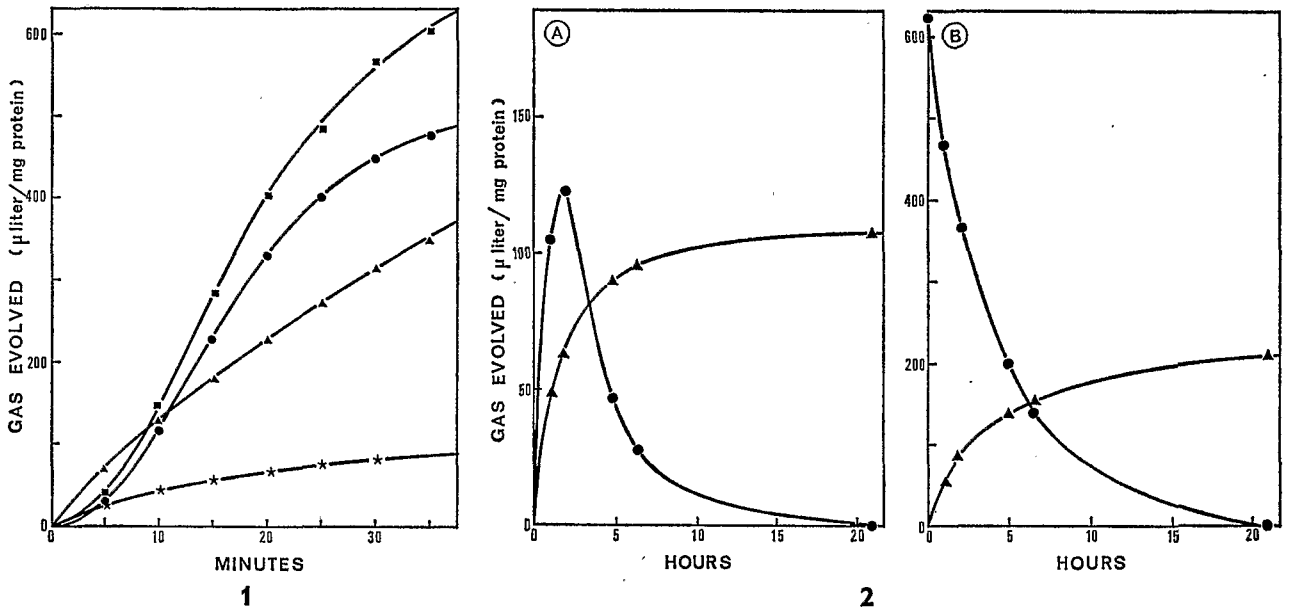


Fig. 1. Manometric measurements of nitrite reduction by crude extract from anaerobically grown cells with PDA, DMPDA, TMPDA or the PMS-ascorbate system as the electron donors, 37°C. Experimental conditions with amines: First side arm: 0.5 M PDA, 0.3 ml; or 0.5 M DMPDA, 0.1 ml; or 0.5 M TMPDA, 0.3 ml. Second side arm: crude extract, 0.04 ml (0.6 mg prot.). Center well: 40% (w/v) KOH, 0.1 ml. Vessel: 0.33 M phosphate buffer pH 7, 1 ml; 0.1 M KNO_2, 0.5 ml; distilled water, up to 3 ml of liquid phase. Gas phase : pure helium. First side arm was tipped into the vessel to allow the chemical reaction, after 15 min gas evolution stopped and the crude extract was tipped from the second side arm. ●——● PDA-NO_2^-; ■——■ DMPDA-NO_2^-; ▲——▲ TMPDA-NO_2^-. Experimental conditions with PMS-ascorbate: side arm: 0.1 M $NaNO_2$, 0.3 ml. Center well: 40% (w/v) KOH, 0.2 ml. Vessel: 0.5 M phosphate buffer pH 6.2, 0.6 ml; 0.1 M sodium ascorbate, 0.3 ml; 1 mM PMS, 0.3 ml; crude extract, 0.2 ml (3 mg prot.); distilled water, up to 3 ml of liquid phase. Gas phase: pure N_2. ∗——∗ PMS-ascorbate NO_2^-

Fig. 2A and B. Gas chromatography measurements of nitrite reduction and nitric oxide reduction by crude extract from anaerobically grown cells, using the PMS-ascorbate system as the electron donor. 125 ml serum flasks incubated at 37°C. 0.5 M phosphate buffer pH 6.2, 6 ml; 0.1 M sodium ascorbate, 3 ml; 1 mM PMS, 3 ml; distilled water, 12 ml (A) or 15 ml (B). Gas phase: pure helium. After thermal equilibration, 3 ml of a 0.1 M $NaNO_2$ solution were added with a syringe in flask A, and 12 ml of pure NO were added with a syringe in flask B. After 1 hr of reaction, 2 ml of crude extract (21 mg prot.) were added with a syringe in each flask. ●——● NO evolution; ▲——▲ N_2O evolution

aerobic and anaerobic conditions of growth. According to Vishniac and Santer (1957) only one aerobic *Thiobacillus* strain has been reported to assimilate nitrate, but all denitrifying strains require ammonium as the assimilatory nitrogen source (Baalsrud and Baalsrud, 1954; Hutchinson *et al.*, 1967).

More recently, Taylor *et al.* (1971) reported an isolate growing anaerobically without ammonium, but this characteristic was not emphasized.

According to Pichinoty and Piéchaud (1968), nitrate reductase A is present when: (1) nitrate reductase and chlorate reductase activities are not additive; (2) the reduction of chlorate is inhibited by 1 mM sodium azide.

These conditions were realized for all extracts of *Thiobacillus denitrificans* "RT" with nitrate either as the terminal electron acceptor (medium DB) or as the nitrogen source (medium B) (see Table 1).

Our data suggest that for *Thiobacillus denitrificans* "RT" nitrate may be reduced by the same protein in both assimilatory and dissimilatory nitrate reductase complex. The idea of only one nitrate reductase has been proposed by Nicholas and Wilson (1964) for *Neurospora crassa*, and by Pichinoty (1966) and Van't Riet *et al.* (1968) for *Aerobacter aerogenes*.

As for *Thiobacillus denitrificans* "Oslo" (Adams *et al.*, 1971), *Aerobacter aerogenes* (Pichinoty, 1963), *Escherichia coli K-12* (Showe and De Moss, 1968) and *Micrococcus denitrificans* (Pichinoty, 1969a; Forget, 1971) nitrate reductase A was localized in a particulate fraction of the cell-free extracts of *Thiobacillus denitrificans* "RT".

As in aerobic conditions growth rates were identical with ammonium or with nitrate as the sole nitrogen source, the assimilatory function of nitrate reductase was not completely inhibited by air in *Thiobacillus denitrificans* "RT".

Anaerobically grown cells suspensions reduced nitrite with thiosulfate as the electron donor and produced, during the beginning of the reaction, a mixture of 48% NO, 40% N_2O and 12% N_2, and nitrogen was the final reaction product. We suggest that this accumulation of intermediates is due to a strong inhibitory effect of nitrite on further steps of the dissimilatory nitrate reduction system.

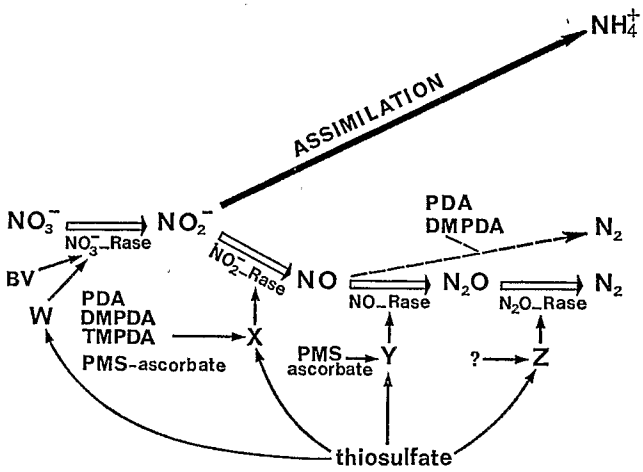
Unlike the isolate of Taylor and Hoare (1971), *Thiobacillus denitrificans* "RT" reduced nitric oxide with thiosulfate as the electron donor to nitrous oxide and nitrogen. The failure of these authors may be accounted for by an inadequate NO concentration in the gas phase when nitric oxide was produced chemically in the Warburg vessels. According to Miyata *et al.* (1969), a 10% NO concentration was necessary to obtain maximum NO reduction rates.

As anaerobically grown cells suspensions reduced nitrous oxide to nitrogen with thiosulfate as the electron donor, we may assume that nitric oxide and nitrous oxide are intermediate products of nitrite reduction by *Thiobacillus denitrificans*. A similar statement was made by Matsubara and Mori (1968) for *Pseudomonas denitrificans*.

Cell free extract from anaerobically grown cells on nitrate produced NO, N_2O and N_2 from nitrite with TMPDA as the electron donor. Nitrous oxide was not detected in nitrite-TMPDA reducing fractions from *Pseudomonas denitrificans* (Miyata and Mori, 1968), *Micrococcus denitrificans* and *Pseudomonas aeruginosa* (Pichinoty, 1969b).

Since our data were in agreement with author's assertion that nitrous oxide was not produced by a chemical reaction between nitric oxide and amines, we may suggest that a nitric oxide-TMPDA reducing activity was present in extracts of *Thiobacillus denitrificans* "RT". However, we were unable to give evidence of enzymatic NO-TMPDA reduction, as nitric oxide was converted into nitrogen by reacting with the phenyl-amino or imino group of the amine, as shown by Miyata and Mori (1968).

This chemical nitrogen production from TMPDA and NO account for the nitrogen evolution detected during enzymatic nitrite reduction with TMPDA as the electron donor, while we were unable to detect any nitrous oxide reducing activity in crude extracts, either with amines, PMS-ascorbate or with NADH as the electron donor systems.



Scheme 1. Hypothetical scheme for a possible mechanism for denitrification by *Thiobacillus denitrificans* "RT". ⇒ Normal denitrification process; ⇢ nonenzymatic reaction; → other enzymatic reaction, or electron transport system. NO_3^--Rase = nitrate reductase; NO_2^--Rase = nitrite reductase; NO-Rase = nitric oxide reductase; N_2O-Rase = nitrous oxide reductase; *W*, *X*, *Y* and *Z* unknown components of electron transport system

Matsubara and Mori (1968) reported that nitrous oxide reductase was lost during disruption of cells of *Pseudomonas denitrificans*, but more recently Payne *et al.* (1971) isolated a nitrous oxide reducing fraction from *Pseudomonas perfectomarinus* grown at the expense of N_2O.

With the PMS-ascorbate system, cell-free extract of *Thiobacillus denitrificans* "RT" strain reduced nitrite to NO+N_2O, and nitric oxide to nitrous oxide.

As a conclusion from the experimental data at the present stage, we can formulate Scheme 1 as a possible mechanism of dissimilatory reduction of nitrate to nitrogen in *Thiobacillus denitrificans* "RT" showing resemblance to the scheme proposed by Matsubara (1970) for *Pseudomonas denitrificans*.

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Dr. J. Baldensperger
Centre ORSTOM, B.P. 1386
Dakar, Sénégal