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# Denitrification by *Azospirillum brasilense* Sp 7

## II. Growth with nitrous oxide as respiratory electron acceptor

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**Abstract.** Nitrous oxide reduction can consistently be demonstrated with high activities in cells of *Azospirillum brasilense* Sp 7 which are grown anaerobically in the presence of low amounts of nitrite. *Azospirillum* can even grow anaerobically with nitrous oxide in the absence of any other respiratory electron acceptor. Nitrous oxide reduction by *Azospirillum* is inhibited by acetylene, amytal and weakly by carbon monoxide. *Azospirillum* converts nitrous oxide to molecular nitrogen without the formation of ammonia. The cells must, therefore, be supplied with ammonia from nitrogen fixation during anaerobic growth with nitrous oxide. When no other nitrogen compound besides nitrous oxide is available in the medium, the bacteria synthesize nitrogenase from protein reserves in about 2 h. Nitrogenase synthesis is blocked by chloramphenicol under these conditions. In contrast, the addition of nitrate or nitrite to the medium represses the synthesis of nitrogenase. Nitrous oxide reduction by *Azospirillum* and other microorganisms is possibly of ecological significance, because the reaction performed by the bacteria may remove nitrous oxide from soils.

**Key words:** Denitrification – Nitrate respiration – Nitrous oxide reduction – Nitrogen fixation – *Azospirillum*

In the accompanying paper (Zimmer et al. 1984), *Azospirillum brasilense* Sp 7 has been shown to grow anaerobically with nitrite as respiratory electron acceptor. Nitrite was converted to $N_2$ without concomitant formation of free $N_2O$. Nitrous oxide was, however, formed when the cells were incubated with $C_2H_2$. The experiments also showed that *Azospirillum* utilized $N_2O$ under anaerobic conditions suggesting that the cells were able to form a dissimilatory nitrous oxide reductase. On the other hand, nitrous oxide utilization could also have been due to nitrogenase which can be synthesized by *Azospirillum*. $N_2O$ is a substrate of this latter enzyme alternative to $N_2$ and $C_2H_2$ (Hardy and Burns 1968). The present investigation will show that $N_2O$ utilization is catalyzed by the dissimilation nitrous oxide reductase and not by nitrogenase. The free energy change of the reaction $N_2O + (H_2) \rightarrow N_2 + H_2O$ is $\Delta G'_0 = -81.6$ kcal/mol = 342.8 kJ/mol (Thauer et al., 1977) and thus high enough to allow growth. It is, therefore, not surprising that most organisms synthesizing $N_2O$ reductase can grow anaerobically with $N_2O$ as sole respiratory electron acceptor (Ingraham 1981; Bryan 1981). The present investigation will show that this is also the case for *Azospirillum brasilense* Sp 7.

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### Materials and methods

*Azospirillum brasilense* Sp 7 (ATCC 29145) was used in all the experiments. Growth conditions were the same as in the accompanying paper. For the $N_2O$-utilization experiments, cells were taken which had been grown anaerobically in the fermenter in continuous culture with malate limiting and with nitrite as the respiratory electron acceptor. The 16 l fermenter contained 5.2 l of the nitrite medium (accompanying paper) and the reservoir the basal medium (accompanying paper) at pH 5.9 containing 5.5 mM $NaNO_2$ and 2.5 mM L-malate. Other data for the continuous culture were pH 7.6, optical density at 560 nm = 0.13, cell number $9.4 \times 10^{10}$ cells/l, dry weight 62.5 mg/l, protein 28 mg/l, growth temperature 30° C, dilution rate 0.042/h and gas flow rate 38 cm$^3$ $N_2$/min. Because the steady state in the continuous culture could be maintained for at least 4 weeks, the same starting material was available throughout the experiments. Under these conditions, *Azospirillum* expressed nitrous oxide reductase with high activity. For the assays (Figs. 2–4; Table 2), *Azospirillum* taken from the fermenter was centrifuged for 10 min at $7{,}000 \times g$ and 20° C under anaerobic conditions to remove nitrite out of the medium and to concentrate the cells 3–6 fold. The bacteria were suspended in the nitrogen free medium which contained L-malate (10 mM) and which was flushed with nitrogen 1 h prior to use. *Azospirillum* was assayed under argon in 7.0 ml Fernbach flasks covered with suba seals and containing 3.0 ml cells (O.D.$_{560\,nm}$ = 0.375/cm). The experiments were started by injecting $N_2O$ (final concentration 16.7 mM in the gas phase) and performed at 30° C in a shaking water bath and for the time indicated in the tables and figures.

Growth of *Azospirillum brasilense* Sp 7 under anaerobic conditions with $N_2O$ as terminal respiratory electron acceptor (Table 1) was performed also in 7 ml Fernbach flasks covered with suba seals and which contained 2.9 ml nitrogen-free medium with 37 mM D-L-malate and 8.5 mM $K^+$-phosphate buffer pH 7.2. The gas phase was argon plus 16.7 mM $N_2O$. The experiments were started by injecting 0.1 ml cell suspensions (10 times concentrated by centrifuging at $12{,}000 \times g$ for 10 min) taken from the continuous culture grown with nitrite. Incubation was performed in a shaking water bath for 24 h and terminated by measuring

the absorbance at 560 nm. Where indicated, the vessels contained: $NH_4Cl$ 20 mM and $C_2H_2$ 5.6 mM in the gas phase.

In a different set of experiments (Fig. 1), $N_2O$-dependent growth of *Azospirillum* was followed under sterile conditions in 1 l flasks containing 350 ml nitrogen free medium with 10 mM L-malate and 55.7 mM $K^+$-phosphate buffer pH 7.2. Cells taken from the fermenter were washed by centrifuging and suspending, and 35 ml of the suspension were used as the inoculum. Incubation was performed at 30°C under argon in two different 1 l flasks. One of them was used for determining $N_2O$-disappearance and $N_2$-formation and the other for measuring growth parameters of the cells. During the assay which lasted for 48 h, the concentration of $O_2$ was less than 0.05% in the gas phase. This experiment was repeated at least five times with reproducible results.

The analytical procedures were the same as in the accompanying paper (Zimmer et al. 1984). $C_2H_2$-reduction was determined by gas chromatography using a flame ionization detector and a Porapak R column. $O_2$ and $N_2$ were measured in a gas chromatograph fitted with a thermal conductivity detector and a molecular sieve 5 Å column with argon as the carrier gas.

**Table 1.** Growth of *Azospirillum brasilense* Sp 7 under anaerobic conditions with $N_2O$ as the terminal respiratory electron acceptor

| Assay condition | Experiment 1 | Experiment 2 |
|---|---|---|
| 1. Gas phase argon anaerobic control | 0.094 ± 0.014 | 0.064 ± 0.005 |
| 2. Gas phase argon + 20 mM $NH_4Cl$ | 0.097 ± 0.010 | 0.073 ± 0.008 |
| 3. Aerobic control (air) + 20 mM $NH_4Cl$ | 2.165 ± 0.049 | 1,450 ± 0.255 |
| 4. Gas phase argon $N_2O$ as the electron acceptor | 0.404 ± 0.049[a] | 0.364 ± 0.065[b] |
| 5. Gas phase argon $N_2O$ as the electron acceptor + 20 mM $NH_4Cl$ | 0.568 ± 0.086 | – |
| 6. Gas phase argon $N_2O$ as the electron acceptor + $C_2H_2$ | – | 0.071 ± 0.003 |

Data mean $O.D._{560\,nm}$ after 24 h incubation. Standard deviations are calculated from 4 repetitions for each experiment. For experimental details see Materials and methods

[a] Corresponding to $2.55 \times 10^8$ cells/ml, assay started with $8 \times 10^7$ cells/ml

[b] Corresponding to $7.4 \times 10^7$ cells/ml, assay started with $3.7 \times 10^7$ cells/ml

## Results

When *Azospirillum* was incubated for 24 h in the 7 ml Fernbach flasks under anaerobic conditions, the increase in turbidity was dependent on the presence of nitrous oxide in both sets of experiments (Table 1). No increase was observed in controls where the gas phase contained only argon and where the medium was supplemented with ammonia. This indicates that the residual $O_2$-concentration in the medium was too low to support respiratory electron transport in *Azospirillum*. When both $N_2O$ and $NH_4^+$; were added to the vessels, turbidity was slightly higher after 24 h than in experiments with $N_2O$ alone. Cell density did not increase when the vessels contained $C_2H_2$ during incubation. Table 1 also indicates that the turbidity increase in the assays with $N_2O$ was $1/4 - 1/5$ of that observed when $O_2$ was the respiratory electron acceptor. Cell number determinations indicated that the cells had multiplied 2–5 times under anaerobic conditions in the presence of $N_2O$ in the gas phase.

The latter was more accurately determined in an experiment where all the growth parameters were followed in a 350 ml batch culture inoculated under argon to which $N_2O$ was added at the times indicated in the figure (Fig. 1a, b). The decrease in $N_2O$-content paralleled with the appearance of molecular nitrogen in the gas phase (Fig. 1a). $N_2$ was the only product of $N_2O$-reduction, and the cells did not excrete ammonia, neither in the absence or presence of methionine sulfoximine which is an inhibitor of glutamine synthetase activity (experiments not documented). The stoichiometry between nitrous oxide disappearance and nitrogen formation approached to unity (Fig. 1a). However, it was difficult to establish the exact ratio. The solubility of $N_2O$ is about 50 times higher than that of $N_2$ in water (Landolt-Börnstein 1923), and the solubility of gases may be different in thick bacterial suspensions and in water. During the 48 h of the assay, cell number, turbidity, protein and also poly-$\beta$-hydroxybutyrate content increased in an almost parallel way (Fig. 1). The specific growth rate was $\mu = 0.065$/h, and the generation time 10.5 h (taken from the protein curve). The increase in the concentration of poly-$\beta$-hydroxybutyrate in the cells (Fig. 1) indicates that respiratory electron acceptors were limiting despite of the presence of $N_2O$ in the vessels. This is in contrast to the situation when nitrite is the respiratory electron acceptor (see accompanying paper). As expected, the cells continuously utilized malate during the assay (Fig. 1a).

$N_2O$-utilization could have been due to dissimilatory nitrous oxide reductase or to nitrogenase. Nitrogenase ($C_2H_2$-reduction) activity could be detected only in those cells which were incubated with $N_2O$ for 4 h under anaerobic conditions after they had been taken out of the fermenter and after they had been freed from nitrite by centrifugation and washing (Fig. 2). $C_2H_2$-reduction in dependence of the $O_2$-concentration shows the typical bell-shaped curve which had been described for $N_2$-fixation by *Azospirillum* and other $N_2$-fixing microorganisms (Döbereiner and De-Polli 1980; Döbereiner 1983). When the cells were assayed directly after the removal from the fermenter and washing without incubation with $N_2O$, no $C_2H_2$-reduction activity could be detected irrespectively of the $O_2$-concentrations in the assays (Fig. 2).

In contrast, $N_2O$-utilization and $N_2$-formation commenced immediately after the removal of the cells out of the fermenter and after washing (Fig. 3). Both $N_2O$-utilization and $N_2$-formation were completely blocked by low amounts of $C_2H_2$ in the assays (Fig. 3). These observations already suggest that $N_2O$-utilization is catalyzed by a dissimilatory nitrous oxide reductase rather than by nitrogenase.

This statement follows even more conclusively from experiments with chloramphenicol (Fig. 4). $C_2H_2$-reduction could be detected not before 2 h after the incubation with $N_2O$ (Fig. 4a). The expression of nitrogenase activity was completely blocked by chloramphenicol, indicating that protein synthesis was required before $C_2H_2$-reduction could start. In contrast, both $N_2O$-utilization (Fig. 4b) and $N_2$-formation (Fig. 4c) were not affected by chloramphenicol at the beginning of the experiment. Later on chlor-

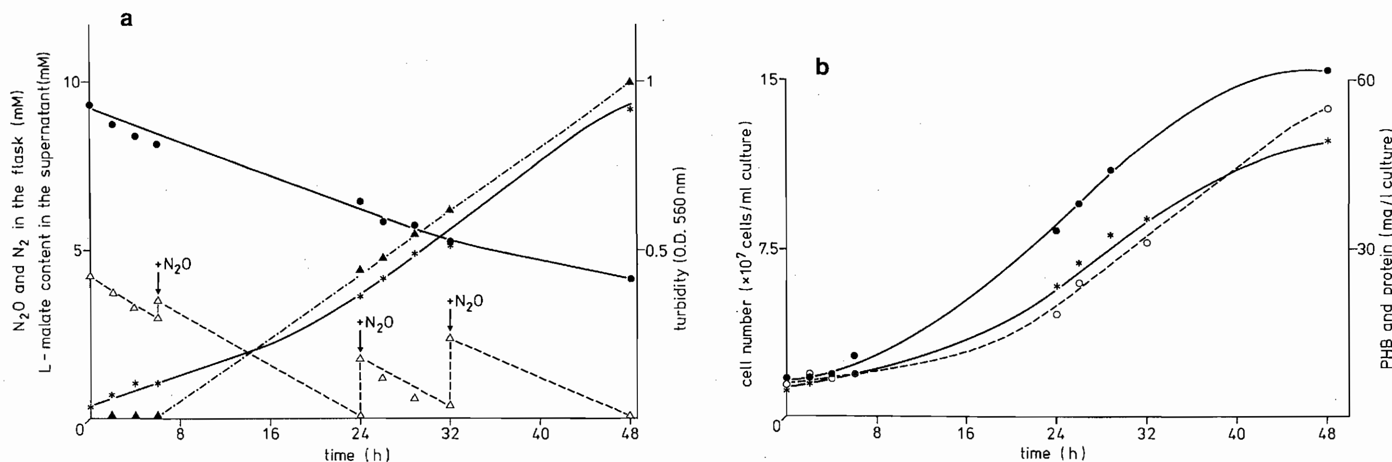


**Fig. 1a, b.** Nitrous oxide dependent anaerobic growth of *Azospirillum brasilense* Sp 7 in batch culture. The experiment was performed in 1 l flasks as described under Materials and methods. △----△ $N_2O$ content in the gas phase (mM); ▲-·-·-▲ $N_2$ content in the gas phase (mM); ●——● L-malate content in the supernatant (mM); ∗——∗ turbidity ($O.D._{560\,nm}$); ●——● cell number ($\times 10^7$ cells/ml culture); ∗——∗ protein (mg/l culture); ○----○ poly-$\beta$-hydroxybutyrate content in the cells (mg/l culture)

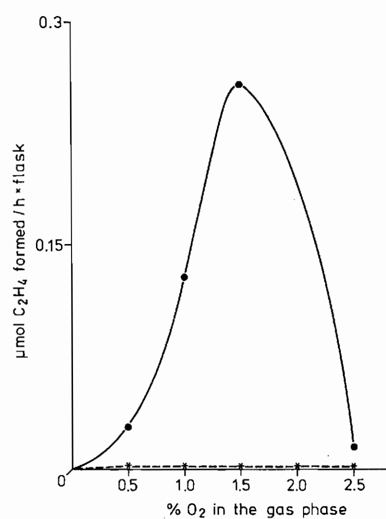


**Fig. 2.** Acetylene reduction by *Azospirillum* Sp 7 in cultures incubated for 4 h with $N_2O$ under anaerobic conditions. After removing from the fermenter, centrifuging and suspending, the cells were incubated for 4 h in the 7.0 ml Fernbach flasks anaerobically with $N_2O$ (16.7 mM in the gas phase). After this time the flasks were evacuated and filled with argon, the different concentrations of $O_2$ as indicated in the abscissa of the figure and $C_2H_2$ (11 mM in the gas phase). $C_2H_2$-reduction was performed for 30 min at 30°C. ●——● $C_2H_2$-reduction; ∗----∗ control, $C_2H_2$-reduction in cells tested immediately after the removal from the fermenter, without incubation with $N_2O$

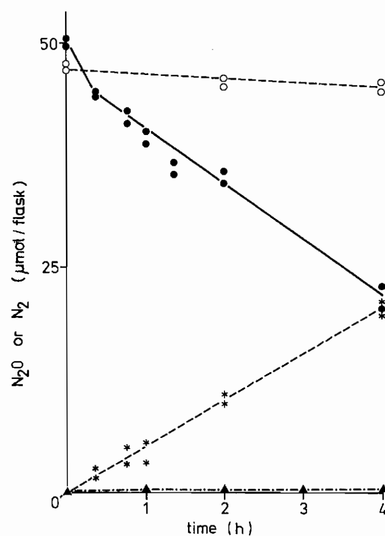


**Fig. 3.** Kinetics of $N_2O$-utilization and $N_2$-formation by *Azospirillum brasilense* Sp 7. The cells (3.0 ml; $O.D._{560\,nm} = 0.375$/cm) were assayed in the 7.0 ml Fernbach flasks as described under Materials and methods. Assay performed without $C_2H_2$ in the gas phase: ●——● $N_2O$-content ln the gas phase of the vessel (mM); ∗——∗ $N_2$-content in the gas phase of the vessel (mM). Assay performed in the presence of 11 mM $C_2H_2$ in the gas phase: ○----○ $N_2O$-content in the gas phase (mM); ▲-·-·-▲ $N_2$-content in the gas phase (mM)

amphenicol progressively affected both $N_2O$-utilization and $N_2$-formation. This observation was not surprising, because dissimilatory nitrous oxide reductase must have had a limited life-time as other enzymes and must, therefore, have been replenished.

In summary, *Azospirillum* reduces nitrous oxide to nitrogen gas under anaerobic conditions catalyzed by a dissimilatory nitrous oxide reductase. Because the only product is $N_2$ and because the cells cannot convert $N_2O$ to ammonia, they must synthesize nitrogenase from protein reserves which is achieved in about 2 h.

The effects of several inhibitors on $N_2O$-reduction are listed in Table 2. $N_2O$-reduction is blocked by $C_2H_2$ but not by $C_2H_4$ or antimycin A. The latter compound may not have been penetrated into the cells. Nitrous oxide reductase is inhibited by amytal and cyanide which has also been described for the enzyme from other organisms (Matsubara 1975). Carbon monoxide affects nitrous oxide reduction only weakly (Table 2) similar as nitrite reduction (accompanying paper).

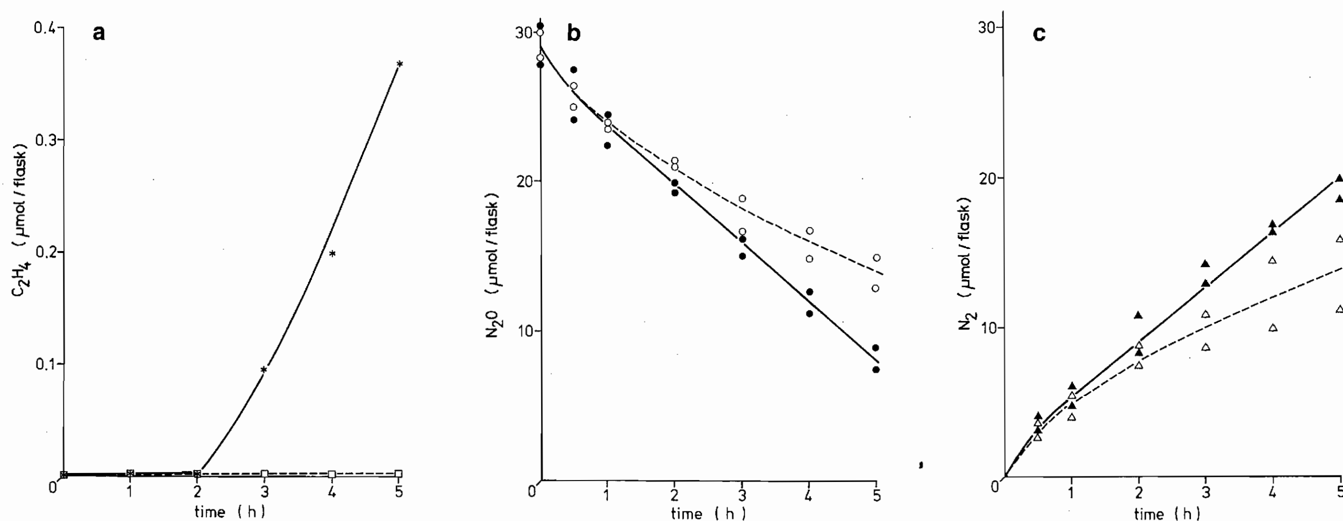


**Fig. 4a—c.** Kinetics of $N_2O$-utilization and $N_2$-formation in the presence or absence of chloramphenicol and time course for the derepression of nitrogenase activity in *Azospirillum brasilense* Sp 7. After removal from the fermenter, centrifuging and suspending, the cells (2.8 ml, $O.D._{560\,nm}$ = 0.625/cm) were incubated with $N_2O$ (16.7 mM in the gas phase). $N_2O$-utilization and $N_2$-formation was determined every h. $C_2H_2$-reduction activity was measured by removing $N_2O$ from the flasks at the times indicated in the abscissa of Fig. 4c and by substituting the gas phase with argon, 11 mM $C_2H_2$ and 1.5% $O_2$. $C_2H_4$-formation was determined after 30 min incubation at 30°C. **a** *——* $C_2H_2$-reduction, − chloramphenicol; □----□ $C_2H_2$-reduction, + chloramphenicol. **b** ●——● $N_2O$-content − chloramphenicol; ○----○ $N_2O$-content + chloramphenicol. **c** ▲——▲ $N_2$-formation, − chloramphenicol; △----△ $N_2$-formation, + chloramphenicol

**Table 2.** Effects of several inhibitors on nitrous oxide reduction by *Azospirillum brasilense* Sp 7

| Inhibitor | Concentration of the inhibitor (mM) | Rate (µmol $N_2O$ utilized/h × mg prot.) | % Activity |
|---|---|---|---|
| 1. − | − | 27.4 | 100 |
| 2. Acetylene | 0.64 | 2.1 | 7.8 |
| 3. Ethylene | 1.30 | 26.7 | 97.6 |
| 4. Antimycin A | 0.25 | 28.1 | 100 |
| 5. Amytal | 0.20 | 5.3 | 19.4 |
| 6. Cyanide | 0.10 | 0.2 | 0.7 |
| 7. Carbon monoxide | 0.83 | 0.5 | 1.8 |
| | 0.16 | 7.1 | 26.1 |
| | 0.016 | 26.2 | 95.8 |

Cells were tested which had been grown in the fermenter with limiting amounts of nitrite. These had been freed from nitrite by centrifuging and washing. The assays were performed in the 7.0 ml Fernbach flasks at 30°C for 1−3 h under argon and $N_2O$ (16.7 mM $N_2O$ in the gas phase). For experimental details see Materials and methods

## Discussion

In the transformations of inorganic nitrogen, *Azospirillum* is one of the most versatile organisms among all bacteria investigated so far, as it can perform all reactions of the nitrogen cycle except nitrification. *Azospirillum* carries out nitrogen fixation, assimilatory reduction, dissimilatory reductions of nitrate and of nitrite as well as nitrate-dependent $N_2$-fixation (see Bothe et al. 1981, 1983b). The present investigation shows that *Azospirillum* can even utilize nitrous oxide for growth under anaerobic conditions. The growth studies and the experiments with inhibitors indicate that nitrous oxide reduction is coupled to energy transformation. The experiments reported here also indicate that nitrous oxide is reduced by a dissimilatory nitrous oxide reductase and not by nitrogenase. As *Azospirillum, Rhizobium* carries out the same reactions of the nitrogen cycle (Rigaud et al. 1973; Daniel et al. 1980). *Rhizobium* has recently been shown also to reduce nitrous oxide (Neal et al. 1983). Anaerobic growth with $N_2O$ has not yet been described for this bacterium.

*Azospirillum* can grow anaerobically with nitrate (Bothe et al. 1981, 1983b), nitrite (accompanying paper) and nitrous oxide (this paper). The conditions for the anaerobic growth with nitrous oxide are different from those with nitrate or nitrite. When nitrate or nitrite are available, *Azospirillum* utilizes these for assimilatory and dissimilatory purposes. Then the bacterium does not perform nitrogen fixation which is apparently more energy consuming than assimilatory nitrate reduction. The addition of nitrate to the medium was shown to repress the biosynthesis of nitrogenase in *Azospirillum* (Bothe et al. 1981, 1983b). In dissimilatory nitrous oxide reduction, nitrous oxide is reduced only to molecular nitrogen and not to ammonia (this communication). Consequently, since no other source for the assimilation of nitrogen is available then, the cells must synthesize nitrogenase from protein reserves. The present paper and the preceeding ones (Bothe et al. 1981, 1983b) have shown that nitrogenase biosynthesis is repressed and derepressed within 2−4 h and that nitrogenase biosynthesis strongly depends on the nitrogen source in the medium. The regulation of nitrogenase biosynthesis is a complex process in the organisms which is not fully understood at present. The experiments by Gauthier and Elmerich (1977), however, showed that glutamine synthetase might play a regulatory

role in nitrogenase biosynthesis in *Azospirillum* as in *Klebsiella*.

Nitrous oxide reductase is generally believed to be a labile enzyme. Nothing is known about the factors regulating the biosynthesis of this enzyme except the fact that anaerobic conditions are prerequisital but not sufficient for the expression of $N_2O$-reduction activity (see accompanying paper). In the present study, nitrous oxide reductase was consistently demonstrated with reproducible activities when *Azospirillum* was grown anaerobically with low amount of nitrite or with nitrous oxide. The biochemical properties of the enzyme are also not known at present. Up till now, a nitrous oxide reductase has only been characterized from *Pseudomonas perfectomarinus* which contains 8 copper atoms per molecule (Zumft and Matsubara 1982). The growth medium used here was not supplemented with copper and the activity of nitrous oxide reduction was not enhanced in cells grown with additional copper (not documented). These findings, however, do not exclude the presence of copper in nitrous oxide reductase of *Azospirillum*. The medium and the glass vessels may have contained trace amounts of copper which may have been sufficient for $N_2O$-dependent growth of *Azospirillum*.

As mentioned in the accompanying paper, *Azospirillum* may be of potential use in practical applications. It can perform either nitrogen fixation or denitrification in association with wheat, depending on the amount of oxygen available in the medium (Bothe et al. 1983a). The present finding that *Azospirillum* can grow anaerobically with nitrous oxide has possibly practical significance, too. Nitrous oxide is a natural agent for the destruction of ozone in the stratosphere and is produced by microorganisms in soils. The amounts of $N_2O$ emitted from soils may be lowered by the activities of denitrifiers like *Pseudomonas denitrificans, Vibrio succinogenes* (see Yoshinari 1980) and others, among which *Azospirillum* is now to be included.

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*Note added in proof*

After submission and acceptance of this manuscript, a paper was published in which the authors also demonstrated growth of *Azospirillum brasilense* with nitrous oxide under anaerobic conditions (Tibelius KH, Knowles R (1984). Uptake hydrogenase activity in denitrifying *Azospirillum brasilense* grown anaerobically with nitrous oxide or nitrate. J Bact 157:84–88