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# Intracellular Location and $O_2$ Sensitivity of Uptake Hydrogenase in *Azospirillum* spp.

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**Uptake hydrogenase activity of *Azospirillum brasilense* in vitro (cell-free extract) was very much more sensitive to $O_2$ than was that of *A. amazonense*, and the $O_2$ pressure optima for uptake hydrogenase activities were 0.01 and 0.4 to 3 kPa for *A. brasilense* and *A. amazonense*, respectively. The addition of superoxide dismutase did not increase uptake hydrogenase activity of *A. brasilense* either in vivo or in vitro. The $O_2$ uptake rates of *A. brasilense* and *A. amazonense* were nearly the same. Inhibition of *A. brasilense* $O_2$-dependent uptake hydrogenase activity by $O_2$ was highly reversible under the conditions tested. $O_2$ also markedly inhibited in vitro methylene blue-dependent uptake hydrogenase activity of *A. brasilense*, and this inhibition was highly reversible. It is concluded that the difference in $O_2$ tolerance of the uptake hydrogenases is not due to a difference in respiratory protection in the two species and may be due to inherent differences in the two enzymes. For the three species, *A. brasilense*, *A. amazonense*, and *A. lipoferum*, almost all the recovered methylene blue-dependent uptake hydrogenase activity was associated with the membrane fraction.**

Like most $N_2$-fixing bacteria, microaerobic $N_2$-fixing *Azospirillum brasilense*, *A. lipoferum* (17), and *A. amazonense* (11) show uptake hydrogenase ($H_2$ uptake) activities (5, 8, 22) capable of recycling $H_2$ evolved by nitrogenase (7). Exogenous $H_2$ supports nitrogenase activities in carbon-starved cells of the three species (9, 14), suggesting that, under carbon limitation, $H_2$ oxidation via uptake hydrogenase provides energy for $N_2$ fixation. The preformed uptake hydrogenase activities in the three species show very different $O_2$ tolerances, whereas their nitrogenase activities are roughly equally sensitive to $O_2$ (8, 10).

As part of a study of the reasons for the different $O_2$ tolerances of uptake hydrogenase in the three species, we report here that such differences occur also in cell-free systems and that they can not be attributed to differences in $O_2$ consumption rate.

## MATERIALS AND METHODS

**Organisms and culture conditions.** *A. brasilense* Sp 7 (ATCC 29145), *A. lipoferum* Sp 59b (ATCC 29707), and *A. amazonense* Y6 (ATCC 35121) were grown in the defined L-malate-containing media previously described (9a, 13). In all experiments, $NH_4Cl$ (1.0 g/liter) was added as a nitrogen source. Batch cultures (900 ml) were sparged with a gas mixture containing $O_2$ and $H_2$ at pressures of about 0.80 and 6.7 kPa, respectively, in $N_2$ (9a, 18).

**Cell breakage and membrane preparations.** Cultures were grown to stationary phase, and 600 ml of cells was harvested at 15,300 × *g* at 4°C for 10 min (9a) under $N_2$ protection unless otherwise stated. The cells were washed once with 50 mM deoxygenated potassium phosphate buffer (DOPB) (pH 7) and transferred to a 50-ml $N_2$-filled centrifuge tube closed with a silicone-reinforced Suba-Seal secured by wire for further centrifugation at 12,100 × *g* (4°C) for 10 min. The pellet was resuspended in 10 ml of DOPB and then transferred by syringe to a silicone-reinforced Suba-Seal-capped glass vial with $H_2$ (101 kPa) as the gas phase. Dithionite (DITH), dithiothreitol (DTT), and phenylmethylsulfonyl fluoride (PMSF) were added to 1 mM final concentration. The cell suspension was then disrupted in a sonicator (9a). The broken-cell suspension (crude extract) was centrifuged to obtain the cell-free extract (CFE) (9a). One half of the CFE was transferred by syringe to a 60-ml (or 100-ml) $N_2$-filled serum bottle and diluted with DOPB for CFE assay. The other half of the CFE was transferred to a 25-ml ultracentrifuge tube and diluted to 20 ml with DOPB for ultracentrifugation to separate the membrane fraction and soluble supernatant. The CFE was centrifuged (L8-M Ultracentrifuge, Beckman Instruments, Inc., Fullerton, Calif.) at 180,000 × *g* at 4°C for 3 h. The supernatant was carefully transferred by syringe to a 60-ml $N_2$-filled bottle and diluted with DOPB for $H_2$ uptake assay. The membrane pellet was resuspended in 7 ml of DOPB by using a glass rod and then carefully transferred by syringe to a tissue homogenizer chilled on ice. The pellet was disrupted for 5 to 10 min under $N_2$ protection. The membrane fraction was then transferred to a 60- or 100-ml $N_2$-filled bottle and diluted with DOPB for $H_2$ uptake assay.

All buffers were freed of $O_2$ by sparging with $N_2$ which had been passed through a $H_2$-reduced Hungate column (9a). The $H_2$ and $N_2$ used were also freed of $O_2$ through the same system. All transfers were carried out under a continuous flow of $O_2$-free $N_2$.

**Assays and analyses.** $H_2$ uptake was determined in a 15-min tritium uptake assay in $N_2$-filled serum vials of 14-ml total volume (containing a 1-ml sample) closed with silicone-reinforced butyl rubber stoppers (18). The assay was started by injection of $H^3H$ (3 kPa) plus $O_2$ (0.1 kPa; 1 kPa for whole cells and crude extract) for $O_2$-dependent $H_2$ uptake activity and $H^3H$ (3 kPa) plus 5 mM methylene blue (MB) (optimal concentration) for MB-dependent $H_2$ uptake activity. The protein contents of samples for $H_2$ uptake were 80 to 90 and 100 to 400 μg/ml for whole cells and CFE, respectively. The amounts of $O_2$ and $H_2$ in sparge gases were determined by gas chromatography (3, 18).

$O_2$ consumption was measured at 30°C by using a Rank oxygen electrode (Rank Brothers, Bottisham, Cambridge, United Kingdom) (4). The vessel contained 3 ml of air-

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TABLE 1. Effects of breakage buffer components and gases on $O_2$-dependent uptake hydrogenase activity in *A. brasilense*[a]

| Gas phase | Breakage buffer | $H_2$ uptake activity (nmol of $H_2$/mg of protein per min) | | % Uptake |
|---|---|---|---|---|
| | | Before sonicating (whole cells) | After sonication (crude extract) | |
| $N_2$ | DOPB | 149.4 ± 6.5 | 11.1 ± 0.5 | 7 |
| $N_2$ | DOPB | 137.8 ± 16.3 | 58.5 ± 8.1 | 42 |
| $H_2$ | DOPB + DITH | 106.9 ± 17.1 | 44.7 ± 1.0 | 42 |
| $H_2$ | DOPB + DTT + PMSF | 102.2 ± 2.0 | 123.7 ± 4.7 | 121 |

[a] For this experiment only, exponential-phase cells were withdrawn from a batch culture and placed in 30-ml $N_2$-filled centrifuge tubes for centrifugation (12,000 × *g* at 4°C for 10 min). The pellet was suspended in DOPB and transferred to a Suba-Seal-closed glass bottle with $H_2$ or $N_2$ as the gas phase for sonication. DITH or DTT plus PMSF was added as required.

saturated PB with concentrated stationary-phase cells or CFE containing 0.3 to 1 mg of protein.

Protein was determined by a modified Lowry method (18) with bovine serum albumin as the standard. Data are means of duplicates for figures and triplicates (plus or minus standard error) for tables unless otherwise indicated.

Superoxide dismutase (SOD) and bovine serum albumin were purchased from Sigma Chemical Co., St. Louis, Mo.

## RESULTS

**Breakage condition.** To select the best conditions for cell breakage, intact cells of *A. brasilense* were sonicated in different breakage buffers and gas phases. After sonication, $H_2$ uptake activity of crude extract was the highest with deoxygenated phosphate buffer (DOPB) (pH 7.0) plus DTT and PMSF as breakage buffer (Table 1), suggesting that these were the best conditions for cell breakage.

**$O_2$ optima for $H_2$ uptake in vitro.** For both *A. brasilense* and *A. amazonense*, CFEs showed significant $O_2$-dependent uptake hydrogenase activities (Fig. 1). The $O_2$ pressure optimum for $H_2$ uptake in CFE of *A. amazonense* was broad and in the range of 0.4 to 3 kPa, and the activity was

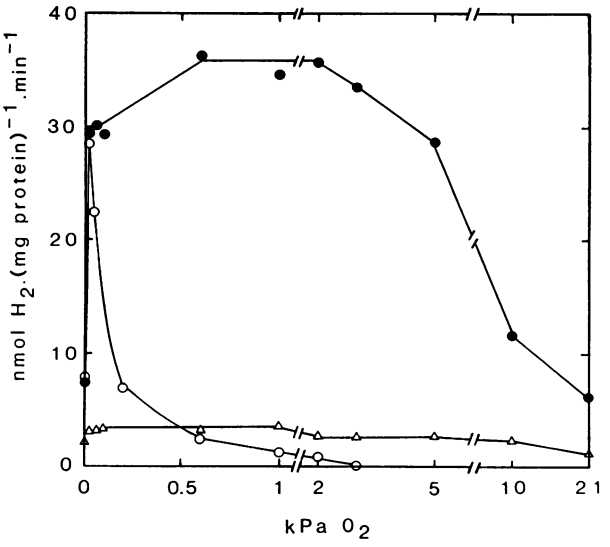


FIG. 1. Effect of $O_2$ on uptake hydrogenase activity in CFEs of *A. brasilense* (○), *A. amazonense* (●), and *A. lipoferum* (△).

TABLE 2. $O_2$ uptake of intact cells and CFE of *A. brasilense* and *A. amazonense*[a]

| Species | System | $O_2$ uptake (nmol of $O_2$/mg of protein per min)[b] |
|---|---|---|
| *A. brasilense* | Whole cells | 55.8 ± 3.8 |
| | Whole cells + malate (10 mM) | 97.7 ± 0.5 |
| | CFE | 7.0 ± 0.1 |
| | CFE + NADH (3 mM) | 41.8 ± 3.5 |
| | CFE + malate (10 mM) | 6.8 |
| *A. amazonense* | Whole cells | 51.2 ± 0.6 |
| | Whole cells + malate (10 mM) | 81.2 ± 0 |
| | CFE | 8.3 ± 0.2 |
| | CFE + NADH (3 mM) | 21.2 ± 0.1 |
| | CFE + malate (10 mM) | 8.0 |

[a] Cells were grown to stationary phase (protein content, 75 to 90 mg/ml).
[b] Data are means (plus or minus standard error) of duplicates except for CFE + malate data, which were single observations.

inhibited 22 and 83% by $O_2$ at pressures of 5 and 21 kPa, respectively. In contrast, the $O_2$ pressure optimum for $H_2$ uptake in CFE of *A. brasilense* was only 0.01 kPa and the activity was inhibited 96 and 100% by $O_2$ at pressures of 1 and 3 kPa, respectively. The data suggest that the uptake hydrogenase in CFE of *A. amazonense* is much more tolerant to $O_2$ than is that of *A. brasilense*, in agreement with our data for whole cells (8). For *A. lipoferum*, the specific activity of the CFE was too low to show a pattern of $O_2$ response, probably because of the very low uptake hydrogenase activity of this species (8). It should be noted that the specific $O_2$-dependent $H_2$ uptake activities of CFEs of all three species were less than 15% of those reported for whole cells (8, 18).

**SOD effect.** It is possible that superoxide free radicals produced by hydrogenase or other electron transport processes were responsible for some of the $O_2$ toxicity (15). $H_2$ uptake activity was therefore determined in whole cells and CFE of *A. brasilense* in the presence of SOD, which was reported to protect hydrogenase against inactivation by superoxide free radicals (15). However, the activity was unaffected by the addition of SOD (0.5 mg/ml in phosphate buffer, pH 7.0) (data not shown), tending to eliminate the possibility that $O_2$ sensitivity of uptake hydrogenase in *A. brasilense* might be due to the effect of such free radicals. That SOD does not prevent the inactivation of hydrogenase has been reported for *Desulfovibrio vulgaris* (21).

**$O_2$ consumption.** It is possible that different $O_2$ tolerances in *A. brasilense* and *A. amazonense* could be due to different $O_2$ consumption rates which provide different degrees of protection from $O_2$. However, the $O_2$ uptake rates in *A. brasilense* and *A. amazonense* were almost the same except in the case of the CFE plus NADH (Table 2). Malate stimulated the $O_2$ uptake rate in whole cells but not in CFEs, possibly because of an NAD limitation following the breakage and dilution of the whole cells. NADH greatly stimulated $O_2$ uptake in the CFEs of both species, indicating that $O_2$ was not limiting. The data therefore suggest that the greater $O_2$ tolerance of *A. amazonense* is not due to a higher $O_2$ uptake rate providing respiratory protection.

**Reversibility of $O_2$ inhibition of $O_2$-dependent $H_2$ uptake activity.** $H_2$ uptake activity of *A. brasilense* in vivo was inhibited markedly by high $O_2$ (Fig. 2, closed circles), in agreement with our earlier report for $N_2$-fixing cultures (8). However, $H_2$ uptake activity in vivo recovered up to 55 to 90% after removal of the $O_2$ present during preincubation

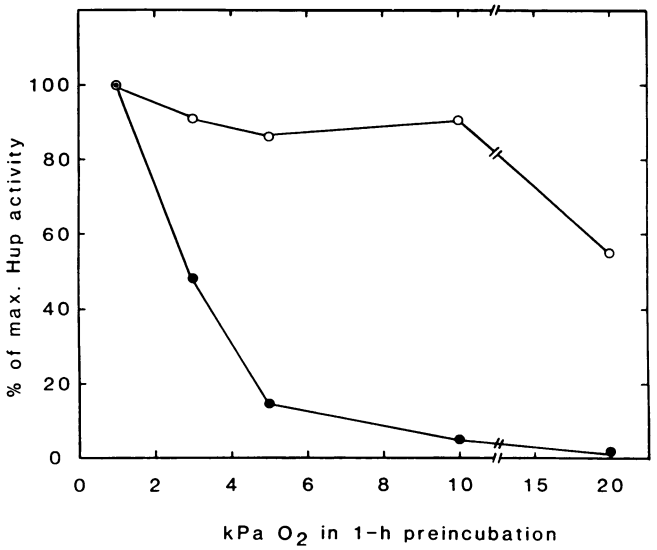


FIG. 2. Reversibility of $O_2$ inhibition of $O_2$-dependent uptake hydrogenase (Hup) in intact *A. brasilense*. Exponential-phase cells were preincubated with different pressures of $O_2$ (1 to 20 kPa) for 1 h. Then, all vials were evacuated (three 15-min evacuations) and backfilled with $N_2$. Each treatment group was divided into two sets for $H_2$ uptake. One set contained $H^3H$ at 3 kPa plus $O_2$ at 1 kPa (○); the other set contained $^3H$ at 3 kPa plus the same $O_2$ pressure as in the preincubation (1 to 20 kPa) (●). Chloramphenicol (100-μg/ml final concentration) was added after the preincubation with $O_2$ to prevent recovery of hydrogenase synthesis during evacuation. The maximum activity was 180.7 nmol of $H_2$ per mg of protein per min.

(Fig. 2, open circles). The reversibility of the $O_2$ inhibition was also observed in CFE of the same species, and the activity recovered up to 73 to 85% after removal of the $O_2$ at least up to the 3 kPa of $O_2$ tested (Table 3). The data suggest that the $O_2$ inhibition of $O_2$-dependent uptake hydrogenase in *A. brasilense* is highly reversible.

**$O_2$ inhibition of MB-dependent activity.** Whether CFE was preincubated with $O_2$ or not, $O_2$ greatly inhibited MB-dependent uptake hydrogenase in CFE of *A. brasilense* (Table 4). $O_2$ at a pressure of 1 kPa also greatly inhibited MB-dependent activity in membrane preparations (data not shown). In either CFE or membrane preparations, MB-dependent activity almost completely recovered after removal of the $O_2$ (5 kPa in CFE; 20 kPa in the membrane fraction) present during preincubation (Table 5), indicating that $O_2$ inhibition of MB-dependent uptake hydrogenase activity of *A. brasilense* is highly reversible.

TABLE 3. Reversibility of $O_2$ inhibition of $O_2$-dependent uptake hydrogenase in CFE of *A. brasilense*[a]

| $O_2$ pressure (kPa) during preincubation | $H_2$ uptake activity (nmol of $H_2$/mg of protein per min) | % Uptake |
|---|---|---|
| 0 | 8.4 ± 1.2 | 100 |
| 0.1 | 8.3 ± 1.9 | 99 |
| 0.3 | 7.1 ± 0.9 | 85 |
| 0.6 | 7.2 ± 0.2 | 86 |
| 1.0 | 6.0 ± 0.8 | 71 |
| 3.0 | 6.1 ± 0.2 | 73 |

[a] Samples were preincubated with $O_2$ at the appropriate pressure at 30°C for 30 min. Then, all the vials were evacuated (four 10-min evacuations) and backfilled with $O_2$-free $N_2$ for $O_2$-dependent (0.1 kPa) $H_2$ uptake assay.

TABLE 4. $O_2$ inhibition of MB-dependent uptake hydrogenase activity in CFE of *A. brasilense*[a]

| $O_2$ pressure (kPa) present | $H_2$ uptake activity (nmol of $H_2$/mg of protein per min) | |
|---|---|---|
| | Without preincubation[b] | With preincubation[c] |
| 0 | 103.9 ± 2.3 | 119.0 ± 14.4 |
| 1 | 4.5 ± 0.5 | 6.0 ± 0.1 |
| 5 | 4.6 ± 0.4 | 7.6 ± 0.9 |
| 20 | 4.6 ± 0.2 | 5.9 ± 0.7 |

[a] Vials containing CFE samples were reevacuated (four 10-min evacuations) and backfilled with $O_2$-free $N_2$ before any treatment.
[b] $O_2$, MB, and $H^3H$ were added at the same time.
[c] Vials were preincubated with $O_2$ at the appropriate pressure at 30°C for 45 min, and then MB and $H^3H$ were added to the vials for MB-dependent $H_2$ uptake assay.

**Intracellular location of uptake hydrogenase.** Since MB is postulated to accept electrons directly from hydrogenase, MB was used to determine the cellular location of the uptake hydrogenase. For all three species, most of the MB-dependent uptake hydrogenase activity was observed in the membrane fractions whereas supernatants showed negligible activity (Table 6), suggesting that uptake hydrogenase in all three species is associated with the membrane. The same patterns were found for $O_2$-dependent uptake hydrogenase activities (data not shown).

## DISCUSSION

For both *A. brasilense* and *A. amazonense*, CFEs showed $O_2$-dependent uptake hydrogenase activities. The $O_2$ tolerance of both species in vitro was very restricted, compared with that of the whole cells (8), and $O_2$ pressure optima were in the range of 0.01 kPa for *A. brasilense* and 0.4 to 3 kPa for *A. amazonense*. However, the patterns of $O_2$ sensitivity in the two species were similar to those in vivo (8), in that uptake hydrogenase of *A. amazonense* was still much more tolerant to $O_2$ than was that of *A. brasilense*. The facts that the in vitro $O_2$-dependent uptake hydrogenase activities were less than 15% of those reported for whole cells (8, 18) and that the $O_2$ tolerance was more restricted in vitro than in whole cells (8) could be due to some interference with the electron transport chain by sonication and to a loss of protective membrane components during breakage. That MB-dependent uptake hydrogenase activities were much

TABLE 5. Reversibility of $O_2$ inhibition of MB-dependent uptake hydrogenase in *A. brasilense*[a]

| Fraction | $O_2$ pressure (kPa) in preincubation | $H_2$ uptake activity (nmol of $H_2$/mg of protein per min)[b] | % Uptake |
|---|---|---|---|
| CFE | 0 | 118.7 ± 1.4 | 100 |
| | 5 | 114.8 ± 1.5 | 97 |
| Membrane | 0 | 170.0 ± 4.0 | 100 |
| | 5 | 170.1 ± 3.1 | 100 |
| | 20 | 162.1 ± 6.4 | 95 |

[a] Vials with samples were reevacuated (four 10-min evacuations) and backfilled with $O_2$-free $N_2$ before any treatment; then, all the vials were preincubated with $O_2$ at the appropriate pressure at 30°C for 30 min and then evacuated (four 10-min evacuations) and backfilled with $O_2$-free $N_2$ for MB-dependent $H_2$ uptake assay.
[b] Data are means (plus or minus standard error) of duplicates for CFE and triplicates for membrane.

TABLE 6. Distribution of MB-dependent uptake hydrogenase of *Azospirillum* spp. in different fractions

| Species | Fraction | Total protein (mg) | Sp act (nmol of $H_2$/mg of protein per min)[a] | Relative activity (nmol of $H_2$/min) | % Uptake |
|---|---|---|---|---|---|
| *A. brasilense* | CFE | 19.24 | 118.7 ± 1.4 | 2,283 | 100 |
| | Membrane | 9.31 | 185.3 ± 6.6 | 1,725 | 76 |
| | Supernatant | 9.86 | 3.1 ± 0.6 | 31 | 1 |
| *A. amazonense* | CFE | 16.12 | 296.8 ± 1.6 | 4,784 | 100 |
| | Membrane | 8.07 | 351.0 ± 3.0 | 2,833 | 59 |
| | Supernatant | 7.93 | 8.7 ± 0.3 | 69 | 1 |
| *A. lipoferum* | CFE | 20.13 | 46.5 ± 1.7 | 936 | 100 |
| | Membrane | 7.83 | 78.6 ± 3.6 | 651 | 66 |
| | Supernatant | 9.52 | 3.7 ± 0.2 | 35 | 4 |

[a] Data are means (plus or minus standard error) of duplicates for CFE and triplicates for membrane and supernatant.

higher than $O_2$-dependent activities in the CFEs of these species supports this hypothesis.

Production of superoxide free radicals ($O_2^-$) is reported to inactivate hydrogenase in *Alcaligenes eutrophus* (15). However, the addition of SOD, which is reported to protect hydrogenase against inactivation by superoxide free radicals (15), did not increase $H_2$ uptake activity in our study, suggesting that the $O_2$ sensitivity of the uptake hydrogenase of *A. brasilense* is not due to an effect of superoxide free radicals or that there might be sufficient SOD already present (6). The $O_2$ uptake rates in *A. brasilense* and *A. amazonense* were almost the same, ruling out the possibility that the greater $O_2$ tolerance of uptake hydrogenase in *A. amazonense* is due to protection by excessive respiratory $O_2$ consumption.

In vivo, $O_2$-dependent uptake hydrogenase activity of *A. brasilense* was completely inhibited by $O_2$ at a pressure of 20 kPa but this inhibition was 57% reversible. This contrasts with an earlier report by Tibelius and Knowles (18), in which hydrogenase in vivo was reported to be irreversibly inhibited by air. This could be due, at least partially, to the different systems used in the assays resulting in different $O_2$ pressures. $O_2$ acts as a reversible inhibitor of anaerobically purified *Bradyrhizobium japonicum* hydrogenase if the sample is exposed to $O_2$ at a low pressure for a short time (2). However, aerobically purified hydrogenases of *B. japonicum* (16) and *D. vulgaris* (20) are very stable in air, and the soluble hydrogenase in *Alcaligenes eutrophus* is actually stabilized by $O_2$ (15).

$O_2$ markedly inhibited MB-dependent uptake hydrogenase of *A. brasilense* in vitro, and the activities of CFE and membrane preparations recovered by more than 95% after removal of the $O_2$. This indicates that $O_2$ inhibition of MB-dependent uptake hydrogenase is almost completely reversible and suggests that hydrogenase purification could be conducted under aerobic conditions. $O_2$ was also reported to be a reversible inhibitor of MB-dependent $H_2$ oxidation in membrane preparations of *B. japonicum*, and the activity continuously increased for a few minutes after the $O_2$ was consumed by respiration (12).

Since MB can accept electrons directly from hydrogenase, the fact that the MB-dependent activity of *A. brasilense* was markedly inhibited by $O_2$ suggests that $O_2$ acts on hydrogenase per se, although the possibility exists that the $O_2$ sensitivity of $O_2$-dependent uptake hydrogenase in *A. brasilense* is partially due to the sensitivity of some components of its electron transport chain (14). We therefore conclude that the differences in $O_2$ sensitivity of the uptake hydrogenases are not due to differences in cellular protection mechanisms and may be due to inherent differences in the enzymes of the two species.

MB-dependent uptake hydrogenase activities in all three species were found to be membrane bound, as in *Azospirillum* sp. strain CC (19). Membrane-bound hydrogenases have been reported for many other microorganisms (1).

## ACKNOWLEDGMENTS

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