# Predominance of hydrogen-utilizing bacteria among $N_2$-fixing bacteria in wetland rice roots

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Heterotrophic bacteria were isolated from wetland soil, rhizosphere soil, root and basal shoot of wetland rice, dryland soil, and root of dryland rice. The isolates were tested for $N_2$-fixing activity and the ability to grow autotrophically under $H_2 + CO_2 + O_2$. $N_2$-fixing bacteria capable of autotrophic growth were found almost exclusively from the rhizosphere and the root of wetland rice. In another experiment, all $N_2$-fixing bacteria isolated from wetland rice root had uptake hydrogenase activity. These findings indicate the predominance of hydrogen-utilizing bacteria among $N_2$-fixing bacteria from wetland rice roots.

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Des bactéries hétérotrophes ont été isolées à partir du sol, du sol rhizosphérique, des racines de riz (riz irrigué et riz pluvial inondé) et de la portion immergée des pieds de riz (riz irrigué). L'activité fixatrice de $N_2$ des souches et leur potentialité de se développer de facon autotrophe sous une atmosphère sans azote ($H_2 + CO_2 + O_2$) ont été testées. Les souches possédant ces deux caractéristiques ont été isolées presqu'exclusivement à partir de la rhizosphère et des racines du riz irrigué. La presque totalité des souches isolées à partir des racines de riz irrigué ont montré une activité hydrogénasique, indiquant la prédominance des formes capables d'utiliser l'hydrogéne parmi les bactéries fixatrices de $N_2$ de la rhizosphère du riz irrigué.

## Introduction

Recently, it was found that some heterotrophic $N_2$-fixing bacteria have the ability to grow chemolithotrophically on $H_2 + CO_2 + O_2$ or $O_2 + CH_4$ (Hanus *et al.* 1979; Pedrosa *et al.* 1980; Malik and Schlegel 1981; Sampaio *et al.* 1981). Watanabe and Barraquio (1979) and Barraquio and Watanabe (1981) found a high proportion of $N_2$-fixing bacteria among heterotrophic bacteria in the root of wetland rice and aquatic plants but not in dryland rice and plants. Because wetland rice soils (Yamane and Sato 1964; Harrison and Aiyer 1916) and wetland rice root (Kimura 1981) evolve $H_2$ gas, it is interesting to know if $N_2$-fixing bacteria in the root of wetland plants (or on the surface of root) have an ability to use $H_2$ and grow autotrophically on $H_2$. Therefore, the ability of aerobic $N_2$-fixing bacteria isolated from dryland soil, wetland soil, dryland rice root, and wetland rice root to grow on $H_2 + CO_2 + O_2$ was examined semiquantitatively. The isolates from wetland soil and rice roots were quantitatively examined for the presence of uptake hydrogenase.

## Materials and methods

### *Soil and plant sampling*

The surface oxidized soil and reduced ploughed soil were collected from a wetland field that has been flooded for more than 7 years at the International Rice Research Institute. Rice plants (IR26) were dug from the soil, and soil adhering to roots was gently removed underwater. The removed soil from the final washing was used for the rhizosphere soil sample. The roots were cut into 3-cm pieces and washed with sterile water six times and then macerated using a Waring blendor. This macerated tissue was used for the histosphere sample. The 3-cm basal tips of shoot which were submerged in the soil and floodwater were cut off, washed, and macerated similarly. This portion served as basal shoot sample.

From the dryland field which was adjacent to the wetland field, where the abovementioned samples were taken, the ploughed soil and dryland rice (IR5) were taken. The preparation of the histosphere sample was similar to that for wetland rice.

The soil and the macerated tissues were diluted serially for microbial counting.

### *Growth conditions*

Dilutions were inoculated on 0.1% tryptic soy agar (Difco) plates and incubated for a week at 32°C. Approximately 100 colonies of bacteria that developed on tryptic soy agar were picked and purified on the same medium. The isolates were inoculated into 3 mL of glucose yeast extract semisolid agar in 10-mL Venoject tubes (Jintan Thermo, Osaka) to test for $N_2$-fixing (acetylene ($C_2H_2$) reducing) activities (Watanabe and Barraquio 1979). The isolates were then grown on agar ($15\ g \cdot L^{-1}$ Difco Bacto-agar) plates containing the $H_2$-uptake medium (De Bont and Leijten 1976) supplemented with $0.05\ g \cdot L^{-1}$ Difco yeast extract. Inoculation to this plate was made by replica plating from the tryptic soy agar master plate.

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on which approximately 10 test strains were grown for 4 days. To ascertain the transfer of the test strains, the replica plate was also made on tryptic soy agar plates. The $H_2$-uptake agar plates were incubated under either 20% $H_2$ + 2% $O_2$ + 5% $CO_2$ + 73% $N_2$ or under the same gas mixture but lacking $H_2$. *Pseudomonas facilis* ATCC 17695 and *P. cepacia* ATCC 25416 were used as reference strains for positive and negative growth, respectively, under $H_2 + CO_2 + O_2$. After 3 weeks of growth at 32°C, the growth under the gas mixture with and without $H_2$ was compared. The strains which could grow better under $H_2 + CO_2 + O_2$ were recorded as being positive for autotrophic growth. The positive ones developed elevated colonies under the atmosphere with $H_2$ but slightly visible and unelevated colonies without $H_2$.

To examine $H_2$-dependent $C_2H_2$ reduction, some strains were inoculated into semisolid (1.75 $g \cdot L^{-1}$ Difco Noble agar) $H_2$-uptake medium with yeast extract but without $NH_4Cl$ and grown under $H_2 + CO_2 + O_2$ for 14 days.

The isolates for quantitative assay of uptake hydrogenase were grown on a tryptone yeast agar slant (5 $g \cdot L^{-1}$ Difco tryptone, 3 $g \cdot L^{-1}$ Difco yeast extract, 0.9 $g \cdot L^{-1}$ $CaCl_2$, 15 $g \cdot L^{-1}$ Difco Bacto-agar) for 48 h at 30°C. The bacterial cells were suspended in 3 mL of $H_2$-uptake medium with 0.05 $g \cdot L^{-1}$ yeast extract and then 0.1-mL aliquots of the suspension were inoculated to 3 mL of the same medium in Venoject tubes. One set of tubes was incubated under 20% $H_2$ + 5% $CO_2$ + 2% $O_2$ + 73% $N_2$ and the other set under the gas mixture without $H_2$. All tubes were kept at approximately 30°C for 10–14 days and $O_2$ concentration in the mixture was measured by thermoconductivity detector gas chromatography every 2 days during incubation. When $O_2$ concentration decreased, $O_2$ was supplemented.

*Assays*

For $C_2H_2$ reduction assays, the glucose yeast extract semisolid agar cultures were incubated for 2–4 days at 30°C. After replacing cotton plugs with rubber serum stoppers, 10% of the air in the tube was replaced with $C_2H_2$. After 24 h of incubation at 30°C, ethylene ($C_2H_4$) content was determined with a flame ionization detector gas chromatograph (Lee *et al.* 1977). The isolates that gave more than 10 nmol $C_2H_4 \cdot 24\ h^{-1} \cdot tube^{-1}$ were regarded as positive in $N_2$ fixation.

For uptake hydrogenase activity, the tubes with preincubated cultures were evacuated and flushed three times with Ar and finally filled with a gas mixture of 1% $H_2$ + 5% $CO_2$ + 2% $O_2$ + 92% Ar. The tubes were kept at 30°C for 24 h. $H_2$ was analyzed with a Hitachi 063 thermoconductivity detector gas chromatograph fitted with a 5 Å (0.5 nm) molecular sieve (1.5 m × 3 mm inside diameter) with $N_2$ as carrier gas at 30 $mL \cdot min^{-1}$. After $H_2$ analysis, the optical density at 450 nm was determined to confirm the autotrophic growth under $H_2 + CO_2 + O_2$ by referring to the growth under the gas mixture without $H_2$.

$H_2$-dependent $C_2H_2$ reduction was measured for 24 h under 80% $H_2$ + 2% $O_2$ + 5% $C_2H_2$ + 13% $N_2$. The controls without $H_2$ were provided.

## Results and discussion

The total bacterial counts and percentage of $N_2$-fixing bacteria which are capable and incapable of autotrophic growth are summarized in Table 1. High percentages of $N_2$-fixing bacteria were found in the root, basal shoot, and rhizosphere of wetland rice, but low percentages were found in the wetland soil, and not in the histosphere of dryland rice. These data confirm earlier reports (Watanabe and Barraquio 1979; Barraquio and Watanabe 1981; Barraquio *et al.* 1982). High percentages of $N_2$-fixing bacteria capable of autotrophic growth under $H_2 + CO_2 + O_2$ were noted exclusively in the rhizosphere and histosphere of wetland rice.

In experiment 2, the percentage of nitrogenase-positive bacteria among dryland soil isolates was high but the average activity was 33 nmol $C_2H_4 \cdot 24\ h^{-1} \cdot tube^{-1}$, and 80% of the positive isolates had activities less than 50 nmol $C_2H_4 \cdot 24\ h^{-1} \cdot tube^{-1}$. Wetland rice root isolates had an average of 270 nmol $C_2H_4 \cdot 24\ h^{-1} \cdot tube^{-1}$, and 33% of the positive isolates had activities less than 50 nmol $C_2H_4 \cdot 24\ h^{-1} \cdot tube^{-1}$.

Lower percentage of $N_2$-fixing bacteria among isolates from the wetland rice root in the second experiment was probably due to the younger growth stage (45 days after transplanting). Barraquio *et al.* (1982) showed that the percentage of $N_2$-fixing bacteria in the root of wetland rice was lower at younger stages of rice growth. This finding is not inconsistent with the result in experiment 2. All cultures which were positive in autotrophic growth but negative in $N_2$ fixation were yellow- or orange-pigmented bacteria or actinomycetes. The actinomycetes were not counted in Table 1. These preliminary surveys are semiquantitative because the judgment of $H_2$ utilization was simply based on visual observation of the growth in the atmosphere with and without $H_2$.

To examine more quantitatively the ability of soil and root isolates to utilize $H_2$, isolation was performed again on tryptic soy agar and the isolated cultures were tested for uptake hydrogenase activity. The results are shown in Table 2. The $N_2$-fixing bacteria were exclusively isolated from the histosphere of wetland rice and all of them were positive in uptake hydrogenase activity. The average uptake hydrogenase activity in uptake hydrogenase positive strains was 1.07 $\mu mol \cdot 24\ h^{-1} \cdot tube^{-1}$ (3 mL of medium). The optical density of $H_2$-grown cultures of all uptake hydrogenase positive isolates was higher than that of the cultures grown without $H_2$. The average of the optical density difference between cultures with and without $H_2$ was 0.06. This difference reflects, most likely, the chemolithotrophic growth during preincubation. Among $N_2$-fixing isolates from wetland rice root shown in Table 2, 60% showed reaction with fluorescent antibody against strain H8 which was described by Watanabe and Barraquio (1979).

In a separate experiment, strain H8 showed uptake hydrogenase activity of 100 nmol $H_2 \cdot min^{-1} \cdot mg$ protein$^{-1}$ in 6 h after 21 days of growth under $H_2 + CO_2$

TABLE 1. Population of heterotrophic bacteria and percentage incidence of $N_2$-fixing bacteria with and without ability to grow autotrophically

| Sample | Heterotrophic bacteria·g dry sample$^{-1}$ | No. of isolates tested | % nitrogenase positive: Autotrophic growth Positive | % nitrogenase positive: Autotrophic growth Negative | % nitrogenase negative: Autotrophic growth Positive | % nitrogenase negative: Autotrophic growth Negative |
|---|---|---|---|---|---|---|
| Experiment 1[a] (wetland) | | | | | | |
| Oxidized soil | $9.2\times10^7$ | 90 | 0 | 0 | 0 | 100 |
| Reduced soil | $2.7\times10^7$ | 51 | 9.8 | 2 | 2 | 86 |
| Rhizosphere soil | $0.9\times10^9$ | 88 | 53 | 3.4 | 3.4 | 40 |
| Histosphere (IR26) | $1.0\times10^9$ | 90 | 71 | 10 | 2.2 | 17 |
| Basal shoot | $1.0\times10^9$ | 88 | 20 | 32 | 3.5 | 41 |
| Experiment 2[b] | | | | | | |
| Dryland soil | $5.0\times10^6$ | 43 | 6.5 | 40 | 4.8 | 48.7 |
| Histosphere of wetland rice (IR5) | $4.1\times10^8$ | 88 | 32 | 26 | 10 | 32 |
| Histosphere of dryland rice (IR5) | $1.1\times10^9$ | 71 | 1.4 | 5.6 | 4.2 | 88.8 |

[a]Plant was at heading stage (75 days after transplanting).
[b]Plant was at the maximum tillering stage (45 days after transplanting).

TABLE 2. Population of heterotrophic bacteria and percentage incidence of $N_2$-fixing bacteria with and without uptake hydrogenase

| Sample (wetland) | Heterotrophic bacteria·g dry sample$^{-1}$ | No. of isolates tested | % nitrogenase positive: Uptake hydrogenase Positive | % nitrogenase positive: Uptake hydrogenase Negative | % nitrogenase negative: Uptake hydrogenase Positive | % nitrogenase negative: Uptake hydrogenase Negative |
|---|---|---|---|---|---|---|
| Oxidized soil | $1.3\times10^8$ | 80 | 0 | 0 | 1.2 | 98.8 |
| Reduced soil | $1.2\times10^7$ | 98 | 0 | 0 | 0 | 100 |
| Histosphere[a] | $4.8\times10^8$ | 100 | 93 | 0 | 1 | 6 |

[a]Plant (IR26) was at heading stage.

When the incubation of uptake hydrogenase assay was prolonged, no decrease in $H_2$ was observed if all of the $O_2$ was consumed. This means that uptake hydrogenase is $O_2$ dependent. The culture of strain H8 grown under the $H_2$-containing gas mixture was reisolated on trypticase soy agar. The reisolate was examined by fluorescent antibody prepared against strain H8. The reisolate reacted with the antibody (unpublished).

$H_2$-dependent $C_2H_2$ reduction was studied using four strains which are regarded dominant among wetland rice root $N_2$-fixing isolates. The bacteria grew on the surface of semisolid agar and the activity was $2 \times 10^2$ nmol $C_2H_4\cdot 24\,h^{-1}\cdot tube^{-1}$. Without $H_2$, no activity was detected.

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# Growth of *Legionella pneumophila* in defined media: requirement fo[r] potassium

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The inorganic ions magnesium and potassium were required for optimal growth of *Legionella pr*
defined medium composed of amino acids and inorganic salts. Optimum growth was obtained at conc
20 μg/mL (80 μ*M*) $MgSO_4 \cdot 7H_2O$ and 150 μg/mL (2 m*M*) KCl. Comparable results were obtained
*pneumophila* as well as with both laboratory-adapted and animal-passed strains.

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potassium. Can. J. Microbiol. **28**: 1055–1058.

Les ions de magnésium et de potassium sont requis par *Legionella pneumophila* pour une croissan
à composition chimique définie composé d'acides aminés et de sels inorganiques. La croissance opt
concentrations d'environ 20 μg/mL (80 μ*M*) $MgSO_4 \cdot 7H_2O$ et 150 μg/mL (2 m*M*) KCl. Des rés
obtenus avec les six groupes sérologiques de *L. pneumophila*, ainsi qu'avec les souches conservées a
qui ont subi un passage chez l'animal.

Several chemically defined media have been described for growth of *Legionella pneumophila* and used to analyze its nutrient requirements (George *et al*. 1980; Pine *et al*. 1979; Ristroph *et al*. 1981; Tesh and Miller 1981; Warren and Miller 1979). Recently, several investigators have begun to examine the effects of inorganic ions present in these defined (or complex) media on the growth of this organism. Using a complex medium, Feeley *et al*. (1979) noticed a toxic effect of NaCl on the isolation of *L. pneumophila* from lung tissue. Smalley *et al*. (1980) described enhanced growth of *L. pneumophila* on a selenium-enriched medium. A comprehensive study by Reeves *et al*. (1981) has recently demonstrated specific metal requirements for growth of *L. pneumophila* based on metal deletion studies and inhibition by metal chelators. On the basis of these findings, they developed a trace metal supplement (including calcium, cobalt, copper, iron, magnesium, manganese, molybdenum, nickel, vanadium, and zinc) for optimal growth of *L. pneumophila* in their defined medium.

Preliminary studies in our laboratory with the defined medium of Warr

findings, demonstrating that bot
sium were required for growth o
medium.

*Legionella pneumophila* stra
group 1), Togus-1 (serogroup 2
group 3), Los Angeles-1 (ser
(serogroup 5), and Chicago-2 (s
R. Weaver (Centers for Disease
were used throughout this stu
strain was obtained in our lab
laboratory strain consecutively
guinea pigs by intraperitoneal
bacterial suspension. Bacteria w
coal yeast extract (CYE) agar fr
peritoneal exudate fluid. The an
considered virulent based on the
pig with $\leq 10^8$ bacteria. Labora
(avirulent) are usually infecti
bacteria. The results presente
Knoxville-1 strain but are repres
other strains tested.

For experimental purposes, ba