Mutants of *Rhizobium japonicum* with Increased Hydrogenase Activity

Abstract. *Some strains of* Rhizobium japonicum *can use hydrogen as an energy source for growth under microaerophilic conditions. Mutant strains have been selected that use hydrogen in the presence of high partial pressures of oxygen. The mutants contain more hydrogenase than the parent strain, both as free-living cells and as bacteroids in nitrogen-fixing soybean root nodules.*

The genetic and environmental factors influencing the expression of the hydrogen uptake system of *Rhizobium japonicum* are of great interest. While reducing dinitrogen to ammonia, nitrogenase catalyzes the adenosine triphosphate–dependent evolution of H_2. In the symbiotic association with soybean, the *Rhizobium* hydrogenase increases the efficiency of nitrogen fixation by oxidizing evolved H_2, resulting in significant increases in plant weight and nitrogen content (*1–3*). Little is known of the factors that regulate the expression of hydrogenase activity in bacteroids. Free-living cells of *R. japonicum* are able to oxidize hydrogen to support chemoautotrophic growth (*4, 5*). In free-living cells, carbon substrates and oxygen prevent formation of the H_2 uptake system, whereas H_2 and CO_2 stimulate hydrogenase synthesis (*6*).

Mutant strains of *R. japonicum* that have lost H_2 uptake activity have been obtained (*3, 7, 8*). We reported earlier the isolation of mutant strains that are hypersensitive to oxygen-mediated repression of the H_2 uptake system (*9*). We now describe the selection of mutant strains that express hydrogen-oxidizing activity in the presence of high concentrations of oxygen. These mutants also have increased hydrogenase activity when they are harvested from soybean nodules.

The strategy used to isolate these mutants was to select for strains able to grow chemoautotrophically in high oxygen. Thirteen oxygen-insensitive mutant strains were obtained in seven selection experiments. In a typical experiment, parent strain SR was cultivated in modified Bergersen medium (*10*), washed, and suspended in autotrophic growth medium (*4*) at approximately 5×10^7 cells per milliliter. The cultures were then incubated at 30°C on a gyratory shaker (140 cycles per minute) in an atmosphere of 75 percent N_2, 10 percent O_2, 10 percent H_2, and 5 percent CO_2. Under these conditions the parent strain cannot grow chemoautotrophically because of the effect of repressing levels of oxygen on the H_2 uptake system. When the cell density increased to 5×10^8 cells per milliliter (after 18 days), dilutions of the cultures were spread on medium lacking carbon substrates, and the plates were incubated in an atmosphere of 68 percent N_2, 17 percent O_2, 10 percent H_2, and 5 percent CO_2. Single colonies obtained after 9 days of incubation at 30°C were streaked on the same medium for purification. In some experiments the starting material was a culture of strain SR treated with the mutagen ethyl methane sulfonate (*8*). All oxygen-resistant mutants retained the parental markers for resistance to streptomycin and kanamycin (*7*).

Mutants obtained by this procedure were able to grow chemoautotrophically in atmospheres containing levels of oxygen that prevent autotrophic growth of the wild-type parent (data not shown). Hydrogen oxidation activity in strain SR and mutant strains SR470, SR473, and SR478 was determined in cultures after subjection to the conditions required for derepression of hydrogenase (Table 1). Hydrogen uptake was completely repressed by 10 percent O_2 in the wild-type strain but not in the mutant strains. The mutant strains also produced more hydrogenase activity than the parent strain when derepressed in 1 percent O_2 (Table 1); these conditions are optimal for expression of hydrogenase in the wild-type strain SR (*9*). Although we have observed some variability in the specific H_2 uptake activity during derepression in 1 percent O_2, in more than ten experiments the oxygen-insensitive mutants always produced more (as much as twofold) hydrogenase activity than the wild-type strain. Other experiments indicate that the three mutants listed in Table 1 are also partially relieved of the normal repression of hydrogenase by carbon substrates. For example, when the strains are derepressed (1 percent O_2) in 10 m*M* succinate, strain SR exhibited no H_2 uptake activity, whereas the mutants had rates ranging from 28 to 38 nmole per hour per 10^8 cells. We tested the oxygen-insensitive mutant strains for hydrogenase activity as bacteroids from soybean nodules. Data presented in Table 1 illustrate that whole bacteroids of strains SR470, SR473, and SR478 contained more than twice as much oxygen-dependent H_2 uptake activity as bacteroids of the parental strain.

The hydrogen oxidation system consists of a membrane-bound hydrogen-activating enzyme and several electron transport components (*11, 12*). To determine the amount of hydrogen-activating enzyme in bacteroids and in free-living

Table 1. Oxygen-dependent H_2 uptake activity by free-living cells and bacteroids of strain SR, SR470, SR473, and SR478. Free-living bacteria were cultivated on solid medium (*6*) and derepressed as described (*8*). After derepression for 18 hours, hydrogenase activity was determined amperometrically (*19*) in the presence of 200 to 240 μ*M* O_2. Activities are expressed as nanomoles of H_2 oxidized per 10^8 cells per hour and are averages from duplicate cultures. Bacteroids were isolated from 32-day-old soybean plants (cultivar Essex) grown in Leonard jars (*20*) in a greenhouse without artificial illumination during August 1982 in Baltimore, Maryland. All other growth conditions and procedures for harvesting of bacteroids were as described (*9*). Activities are expressed as micromoles per milligram of protein per hour and are averages of triplicate assays.

Strain	Free-living cells		Bacteroids
	10% O_2	1.0% O_2	
SR	< 1.0	69.1	3.2
SR470	154.0	90.8	6.9
SR473	106.4	106.2	6.8
SR478	113.4	104.4	8.0

Table 2. Methylene blue–dependent H_2 uptake by membranes prepared from strains SR, SR470, SR473, and SR478. Free-living cells were cultured in modified Bergersen medium (*10*) and derepressed (*8*); bacteroids were prepared as described in Table 1. Cells of both types were broken in a French pressure cell as described (*21*), and cell debris was removed by centrifugation at 10,000*g* for 40 minutes. Membranes were isolated from the supernatant by centrifugation at 34,000*g* for 120 minutes, and the membrane pellet was washed and isolated by centrifugation at 34,000*g* for 40 minutes. After cell breakage the membranes were maintained in an atmosphere of 100 percent hydrogen to prevent inactivation of hydrogenase by oxygen. Methylene blue–dependent hydrogen uptake was determined amperometrically (*19*) under strict anaerobic conditions; the absence of O_2 was verified with an oxygen electrode. The assay mixture contained, in 4.8 ml, 240 μmole of potassium phosphate (*p*H 7.0), 12 μmole of $MgCl_2$, 500 nmole of methylene blue, 37.7 nmole of H_2, 60 to 125 μg of protein from the membrane preparation, and 0.5 μmole of dithionite. Activities are expressed as micromoles of H_2 oxidized per hour per milligram of protein and are averages of triplicate assays.

Strain	Source of membranes	
	Free-living cells	Bacteroids
SR	8.9	4.8
SR470	21.2	23.3
SR473	15.7	27.5
SR478	20.4	29.8

cells of each strain, membranes were prepared and methylene blue–dependent H_2 uptake activity was measured, methylene blue being an excellent electron acceptor for purified rhizobial hydrogenase (*13*) (Table 2). By this criterion, free-living cells of the mutant strains contained significantly more hydrogenase activity than the parent strain. Bacteroids of the oxygen-insensitive strains contained five to six times as much H_2-activating enzyme as bacteroids of the wild-type strain (Table 2).

The isolation of *R. japonicum* mutant strains that synthesize large amounts of hydrogenase as bacteroids in soybean nodules is of potential agricultural significance. As strains of *R. japonicum* with enhanced nitrogenase activity are developed (*14*), it may be helpful to increase their hydrogenase activity correspondingly. In addition, the method we used to select oxygen-insensitive mutants may be useful for increasing the hydrogen-oxidizing activity of *Rhizobium* strains having naturally low H_2 uptake activity.

Mutant strains that express hydrogen-oxidizing activity in the presence of high oxygen also should be useful in answering some basic questions concerning the regulatory role of oxygen in *Rhizobium*. Symbiotic characteristics of *Rhizobium* that are expressed in free-living culture only when the cells are subjected to low oxygen include elevated heme-synthesizing activity (*15*) and differentiation into bacteroid-like morphology (*16*). In addition, hydrogenase (*5, 6*), nitrogenase (*17*), and cytochromes similar to those detected in bacteroids (*15, 18*) are expressed by free-living cells only under low oxygen.

The mutant strains that have higher hydrogenase activity in culture also have higher activity in nodules, suggesting that common elements are involved in regulating the H_2 uptake system in free-living cells and bacteroids.

DAVID MERBERG
ROBERT J. MAIER*

Department of Biology,
Johns Hopkins University,
Baltimore, Maryland 21218

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22. Supported by grant 59-2243-0-1-435-0 from the United States Department of Agriculture. This is contribution 1201 from the Department of Biology, Johns Hopkins University.
* To whom correspondence should be addressed.

29 November 1982; revised 28 January 1983

Crystallization and X-ray Analysis of Stemphyloxin I, a Phytotoxin from *Stemphylium botryosum*

Abstract. *Certain isolates of the plant-pathogenic fungus* Stemphylium botryosum *produce a phytotoxin, stemphyloxin I. This toxin ($C_{21}H_{34}O_6$) was crystallized and its structure was determined by x-ray crystallography to be a β-ketoaldehyde* trans-*Decalin. This compound is a highly unusual natural product. Iron (Fe^{3+}) controls production of toxin by this fungus. Furthermore, iron reacts with the toxin to yield a colored product which aids in its detection on chromatograms and in its quantitative estimation by colorimetry.*

Phytopathogenic fungi are commonly able to produce some or all of the disease symptoms in their respective host plants by means of one or more phytotoxins that they produce (*1*). The foliage blight disease of tomato caused by *Stemphylium botryosum* walr. f. sp. *lycopersici* is no exception (*2*). Culture filtrates of this fungal pathogen have been reported to contain a phytotoxin, stemphyloxin I, that is capable of producing the necrotic and chlorotic blighting symptoms associated with this disease (*3*). Stemphyloxin I is bioassayed by placement of a few microliters onto a puncture wound made on a leaf blade. After 14 hours, measurements are made on the size of the necrotic lesion that develops. Stemphyloxin I at concentrations between 10 and 250 μg/ml exhibits differential toxicity toward various plants, with tomato being the most sensitive and no lesions being produced on barley (*Hordeum sativum* L.) (*3*). One can obtain a more sensitive measure of toxin activity by following the incorporation of ^{14}C-labeled amino acids into protein. White or green tomato cell suspensions are completely suppressed in amino acid incorporation in the presence of 1 μ*M* toxin (*3*).

Few compounds that are classified as phytotoxins have ever been crystallized and subsequently subjected to structural analysis by x-ray crystallography (*1*). We have recently obtained crystals of stemphyloxin I suitable for x-ray analysis. Stemphyloxin I (80 mg) was prepared by silica gel H column chromatography (*3*). The toxin preparation was dried under N_2 and dissolved in 1 ml of 70 percent ethanol. The transparent yellowish solution was transferred onto a watch glass (50 mm in diameter), which was placed in an open petri dish. Distilled water was added up to a final ethanol concentration of 60 percent, and then 50 μl of xylene was added. Crystals appeared during incubation of this ethanolic solution at 4°C for 16 hours (*4*). The crystals were dried under N_2, transferred to a stoppered vial, and kept at 4°C until subjected to x-ray analysis (*5*). They were birefringent under the polarizing microscope and stable for at least 9 months at 4°C. A phasing model was achieved, after some difficulty with the use of direct methods (*6*). Block-diagonal, least-squares refinements with anisotropic heavy atoms and isotropic hydrogens have converged to a standard crystallographic residual of 0.0505 (*7*).

Stemphyloxin I is a highly functionalized β-ketoaldehyde *trans*-Decalin (Fig. 1). It does not closely resemble previously reported metabolites of the plant kingdom. Furthermore, we know of no other reported phytotoxin that has a β-ketoaldehyde functionality. In fact, β-ketoaldehydes are quite rare among natural products, although aliphatic enolized β-ketoaldehydes are major constituents used for defense by the termite *Rhinotermes hispidus* (*8*).

Although the structure of stemphyloxin I is highly substituted with methyl groups, it does not seem to obey the rule