Two genes involved in the regulation of nitrogenase activity, draT and draG, were cloned and found to be contiguous on the Azospirillum brasilense chromosome. The nifH gene, encoding dinitrogenase reductase, is near to draT with an intervening gap of 1.9 kb. The organization of these genes in Azospirillum lipoferum and Rhodospirillum rubrum is similar, but nifH and draT are separated by only 400 bp in the organisms. A. brasilense draTG is very similar to draTG in R. rubrum with 91.8% similarity and 85.3% identity at the amino acid level. Apparently A. brasilense uses the normal ATG initiation codon for draT, and draG. The genes for A. brasilense were able to restore function to appropriate mutants of R. rubrum. The heterologous expression of A. brasilense draTG in R. rubrum was not fully normal, as it responded more slowly to darkness and more quickly to ammonia than wild type cells. Our mutational analysis of the draTG region of A. brasilense confirms the function of these genes in the regulation of nitrogenase activity, but it also revealed minor but demonstrable differences in the control systems of R. rubrum and A. brasilense.

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Two genes involved in the regulation of nitrogenase activity, \(\text{draT}\) and \(\text{draG}\), were cloned and found to be contiguous on the \(A.\ brasilense\) chromosome. The \(\text{nifH}\) gene, encoding dinitrogenase reductase, is near to \(\text{draT}\) with a 1.9-kb intervening gap and is divergently transcribed. Similar organization of these genes has been found in \(A.\ lipoferum\) and \(R.\ rubrum\), but in the latter organism \(\text{nifH}\) and \(\text{draT}\) are separated by only 400 bp.

\(A.\ brasilense\) \(\text{draTG}\) is extremely similar to \(\text{draTG}\) of \(R.\ rubrum\) at both amino acid and nucleotide levels, but the similarity was lower than that of their \(\text{nifH}\) genes; they showed 91.8% similarity and 85.3% identity at the amino acid level. Particularly in \(\text{draT}\), there seem to be several regions of extreme conservation, probably reflecting the involvement of those regions of the gene product in its catalytic function or its regulation.

\(R.\ rubrum\) \(\text{draT}\) has the unusual initiation codon TTG, which was verified by determination of the N-terminus of DRAT from that organism. This initiation codon is relatively rare in \(E.\ coli\), and it usually supports poor translation of the gene, which is consistent with the low amount of DRAT in cells. The start codon of \(A.\ brasilense\) \(\text{draT}\) is less clear. We propose that it uses a normal ATG as initiation codon, preceded by a Shine-Dalgarno sequence of GAGAG, but there are two other possibilities. A TTG codon is located at the tenth position, but is not preceded by a reasonable Shine-Dalgarno sequence. More interesting, a comparison with the \(R.\ rubrum\) sequence suggests that the 17th codon of the \(A.\ brasilense\) sequence might be the start site. CTG, like TTG, is typically a leucine codon, but it has not previously been identified as a start codon. Since \(A.\ brasilense\) DRAT has not been purified and its N-terminus is unknown, we cannot reach a firm conclusion about the start codon.

\(\text{draG}\) appears to use ATG as an initiation codon, and it can encode a protein of 32.4 kDa, consistent with the characterization of the purified protein. The sequence also revealed the first 42 nucleotides of an ORF immediately downstream of \(\text{draG}\) that was very similar to a similarly positioned ORF of \(R.\ rubrum\). In \(R.\ rubrum\), the product of this ORF appears to have a secondary role in the regulation of the DRAG/T system. The sequence comparison of the \(\text{draTG}\) to GenBank showed similarity only for \(R.\ rubrum\); it was not similar to the ADP-ribosyltransferase of bacterial toxins.

The functionality of the identified genes was shown both by mutational analysis in \(A.\ brasilense\) and by their ability to complement appropriate mutants of \(R.\ rubrum\). The \(\text{dra}\) mutants behaved rather similarly to those described in \(R.\ rubrum\). In particular, the ability of a \(\text{draC}\) mutant to display active nitrogenase upon derepression, and to respond irreversibly to ammonium, strongly suggests that DRAT activity itself is subject to post-translational regulation as seen in \(R.\ rubrum\).
The expression of the *A. brasilense* dra genes in *R. rubrum* revealed several other interesting features of the system. The ability of DRAT/C from *A. brasilense* to respond to cycles of darkness/light indicates sufficient similarity to the *R. rubrum* products that they still interact with the light/dark signal transduction pathway. Although there have been several studies of signal transduction to this system, including the effects of energy charge, pyridine nucleotide pools and amino acid pools, neither the small molecules nor protein factors involved are known.

Residual nitrogenase activity was observed in wild type and dra mutants of *R. rubrum* after a shift to ammonia or darkness, but no residual activity was found in *A. brasilense* after ammonia addition. *R. rubrum* transconjugants containing *A. brasilense* draTG genes also displayed significant residual activity. This suggests that the residual activity probably can be attributed to other factors in *R. rubrum*, rather than a specific difference in the DRAG system. Consistent with this, no residual nitrogenase activity has been reported in ammonia-treated *K. pneumoniae* when draTG genes from either *R. rubrum* or *A. lipoperox* were expressed in that organism. Higher residual nitrogenase activities (20-30%) in *R. rubrum* transconjugants exposed to darkness or ammonium chloride may reflect greater DRAG accumulation (multicopy of draC gene) or inappropriate regulation of the heterologous DRAG.

The heterologous expression of *A. brasilense* draTG in *R. rubrum* was not completely "normal," since it showed a slower response to darkness and a faster response to ammonia than did wild-type *R. rubrum*. *A. brasilense* itself shows a relatively fast response to ammonia, so that then DRAG regulatory systems in the two organisms are not identical.

Whereas no hybridization of a nifJ probe has been reported in the region between draT and nifH, a putative NtrA-dependent promoter has been described upstream of nifH which would be transcribed toward dra. However, *A. brasilense* strains UB5 and UB6, containing insertion mutations in the region between draT and nifH, showed no detectable effect on nitrogenase or its regulation by DRAG/T.

Our mutational analysis of the draTG region of *A. brasilense* confirms the function of these genes in the regulation of nitrogenase activity. It also demonstrates that the draTG genes are not essential for growth, nor are there duplicated genes or redundant functions in this organism. While the general behavior of the DRAG/T system in *A. brasilense* is similar to that seen in *R. rubrum*, there are a number of interesting differences in the timing and extent of the regulatory response that make the comparative analysis of the two systems important.

The project will continue to support the investigation of the control system for nitrogenase in *Azospirillum* spp. as described in accompanying reprints and the paper that will be submitted soon by Zhang, Burris and Roberts.

Progress has been slow in our efforts to determine the kinetics of N2 reduction and protein reduction by nitrogenase, but we hope that continuing difficulties with the rapid reaction equipment can be resolved so that the quantitative time-course of reduction of N2 and H+ can be established more accurately.
We have confirmed that *Acetobacter diazotrophicus* grows on N\textsubscript{2} at a pH below 3, whereas most nitrogenases are inoperative below pH 5. The organism utilizes a variety of substrates, but it metabolizes glucose and acetate particularly rapidly. Again we have been unable with varied methodologies to demonstrate any exchange reaction accompanying the fixation of N\textsubscript{2} by *Azotobacter vinelandii*.


Publications:

R. H. Burris. Acetylene Reduction to Measure Biological Nitrogen Fixation. Current Contents [Citation Classics] 31, #38, 18 (Life Sciences) and 21, #38, 18 (Agriculture, Biology & Environmental Sciences) (1990).


