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Regulation of Nitrogenase in the Photosynthetic Bacterium *Rhodopseudomonas capsulata* as Studied by Two-Dimensional Gel Electrophoresis

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By using two-dimensional electrophoresis, five putative soluble *nif* gene products were identified, and the regulation of *nif* gene expression in *Rhodopseudomonas capsulata* was investigated. Expression of *nif* was repressed by ammonia and atmospheric concentrations of oxygen. Deprivation of molybdenum caused an interesting pattern of partial repression of *nif* gene expression that was not relieved by tungsten. These results are discussed in relation to the better understood system of *nif* regulation in *Klebsiella pneumoniae*.

The identification of *nif* genes and *nif* gene products and regulation of the expression of the *nif* genes are well advanced for *Klebsiella pneumoniae*. The use of two-dimensional polyacrylamide gel electrophoresis for the analysis of Nif$^-$ strains together with the availability of a large number of genetically well-characterized strains and various biochemical assays have allowed the determination of the physical characteristics and probable biochemical function for many of the protein products of the *nif* genes (2).

By using the two-dimensional electrophoresis technique, the regulation of *Rhodopseudomonas capsulata nif* genes by ammonia, oxygen, and the availability of Mo was examined. Two-dimensional electrophoresis was carried out by using a modification of the methods of O'Farrell (15) and Iborra and Buhler (9). Culture samples were collected by centrifugation at 12,000 × *g* for 20 min and resuspended in 0.125 M Tris, pH 8.0, and an extract was prepared by sonication (7). The soluble protein fraction was obtained by centrifugation at 100,000 × *g* for 90 min. Isoelectric focusing was carried out with an LKB Multiphor with slab gels containing 5% acrylamide–8.4 M urea–2% ampholines (pH range, 3.5 to 10, obtained from LKB). After 1 h of prefocusing, 50 to 80 μg of protein in 6.7 M urea–1.5% Triton X-100–3% β-mercaptoethanol were loaded on the gel and focusing was allowed to run for 2 h at 1,000 V. Bands of 1-cm width were then cut and equilibrated in 2% sodium dodecyl sulfate. Sodium dodecyl sulfate-gel electrophoresis in the second dimension was performed as described by Laemmli and Favre (11), with a separation gel of 10% acrylamide and a stacking gel of 4.5% acrylamide (height, about 1 cm). The bands from the first dimension were loaded on top of the stacking gel and covered with 2% agarose containing 0.1% sodium dodecyl sulfate. After migration, the gels were fixed for 30 min in 50% trichloroacetic acid and then stained overnight in 0.05% Coomassie brilliant blue R250–10% trichloroacetic acid–7.5% isopropanol–0.15 M KCl, prepared fresh each day. Trichloroacetic acid (10%) was used for destaining.

The structural nitrogenase proteins Mo-Fe (dinitrogenase) and Fe (dinitrogenase reductase) were positively identified by electrophoresis (Fig. 1) of the purified proteins isolated as previously described (7). We have tentatively identified two other soluble *R. capsulata nif* gene products by comparing the two-dimensional patterns obtained with high-speed supernatants of cultures grown under a variety of conditions. The molecular weights and isoelectric points of these putative five *nif* gene products are shown in Table 1. DNA hybridization studies indicate a high degree of similarity between the *nif* structural genes of *R. capsulata* and *K. pneumoniae*, but very little similarity in the genes that may code for other *nif* gene products (17). Thus, positive identification of *nif* gene products other than nitrogenase will require the two-dimensional pattern analysis and biochemical characterization of specific Nif$^-$ mutants.

Nitrogenase-regulatory mutant. The two-dimensional gel technique may be used to identi-

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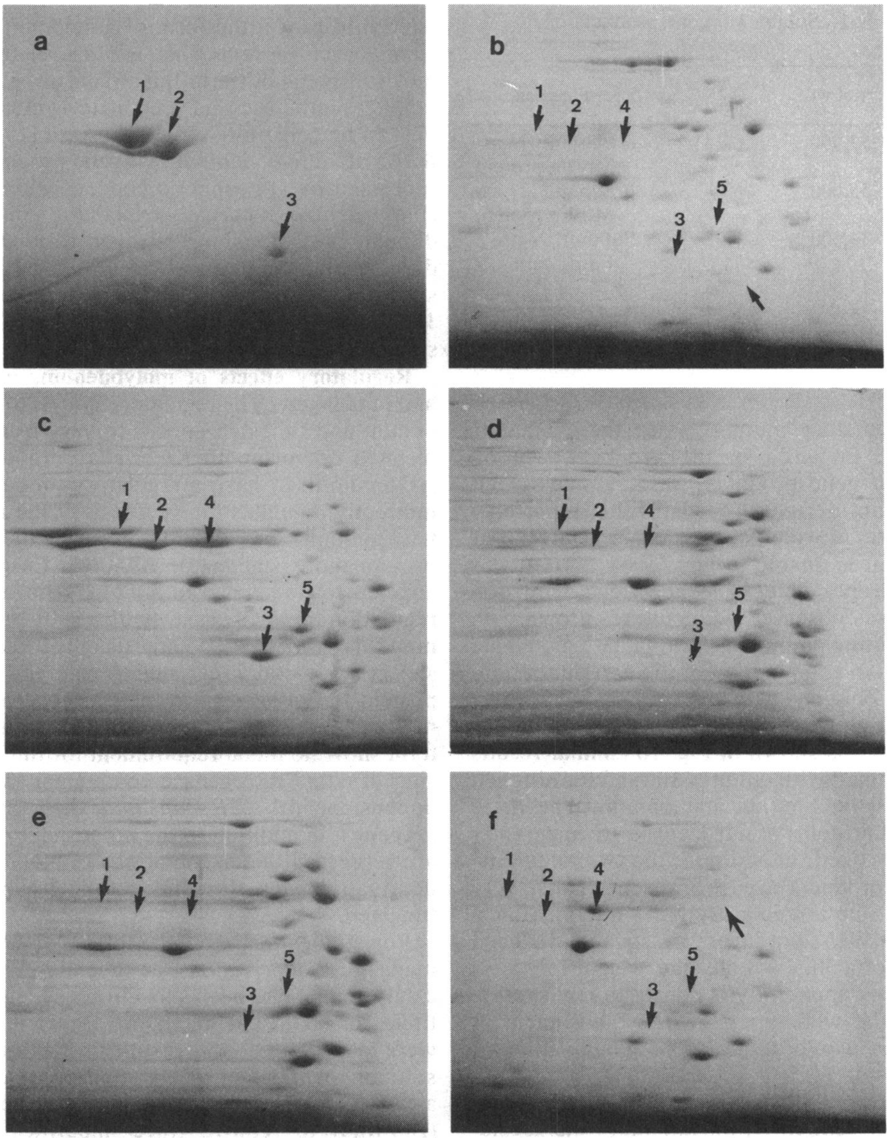

FIG. 1. Two-dimensional gel electrophoresis patterns obtained under various culture conditions. *R. capsulata* strains B-10 and W-15, a Nif⁻ mutant derived from B-10, were obtained from the Photosynthetic Bacteria Group, Department of Microbiology, Indiana University, Bloomington, and were cultured in 1-liter batches as previously described (7). (a) Two-dimensional gel electrophoresis of the purified nitrogenase proteins. A 35-μg amount of the Mo-Fe protein (dinitrogenase) and a 5-μg amount of the Fe protein (dinitrogenase reductase) were applied. The numbered arrows indicate the various subunits as shown in Table 1. (b) Soluble protein pattern of the Nif⁻ mutant W15. The numbered arrows indicate the normal location of the *nif* gene products shown in Table 1. The unnumbered arrow indicates the position of a protein apparently constitutively synthesized in the wild type. (c) Soluble protein pattern of wild-type cells cultured on lactate (30 mM) and glutamate (7 mM). The numbered arrows indicate the various *nif* gene products as shown in Table 1. (d) Soluble protein pattern of wild-type cells cultured on lactate (30 mM) and ammonia (7 mM). The numbered arrows indicate the normal location of the *nif* gene products shown in Table 1. (e) Soluble protein pattern of wild-type cells cultured in the dark on lactate (30 mM), glutamate (7 mM), and oxygen. The numbered arrows indicate the normal location of the *nif* gene products shown in Table 1. (f) Soluble protein pattern of wild-type cells grown on lactate (30 mM) and limiting ammonia (2 mM) under molybdenum-deficient conditions. The numbered arrows indicate the normal location of the *nif* gene products shown in Table 1. The unnumbered arrow indicates the position of a protein apparently constitutively synthesized under Mo-sufficient conditions.

TABLE 1. Soluble *nif* gene products of *R. capsulata*

Spot no.	Mol wt	Isoelectric point	Function
1	59,500	6.25	α Subunit, N_2ase Mo-Fe protein
2	55,000	6.0	β Subunit, N_2ase Mo-Fe protein
3	33,500	5.35	Subunit, N_2ase Fe protein
4	55,000	5.65	—[a]
5	38,400	5.14	—

[a] —, Unknown.

fy *nif* regulatory mutants since these mutants would be impaired in the production of all known *nif* gene products. W15, a single-point Nif⁻ mutant derived from B-10 that reportedly can utilize a wide variety of N sources for growth but is unable to grow with N_2 (19) was examined. No detectable acetylene reduction activity was observed with cultures grown with either limiting ammonia or on lactate-glutamate medium. An examination of the two-dimensional pattern obtained with an extract of W15 grown on lactate-glutamate (normally derepressing for nitrogenase) is shown in Fig. 1b (similar results were obtained with cultures starved for nitrogen by growth on limiting ammonium). The three structural proteins of nitrogenase are apparently not synthesized; in addition, the two additional *nif* gene products are also absent. Thus, W15 appears to be a regulatory Nif⁻ mutant. (Interestingly, W15 produces drastically reduced amounts of a 30,500-molecular-weight (pI, 5.22) protein that apparently is constitutively synthesized in the wild type.) Firm establishment of this point would require the examination of revertants of W15.

Regulation of nitrogenase expression by ammonia and oxygen. In *K. pneumoniae*, the regulation of *nif* gene expression by oxygen, ammonia (or other nitrogenous compounds), and probably temperature, is exerted at the level of the RLA operon. Thus, all *nif* genes are coordinately expressed or repressed (2). At present, it is not known whether the same system of regulation operates in the control of *nif* gene expression in photosynthetic bacteria. In common with the system described for *Klebsiella* spp., all identified *nif* gene products were repressed under atmospheric oxygen or with excess ammonia in the medium. Figure 1c shows the distribution of soluble proteins from a culture grown under conditions (lactate and glutamate) allowing *nif* gene expression (8). The three structural *nif* gene products and the two other presumed *nif* gene products were not expressed (Fig. 1d) when *R. capsulata* was grown with an excess of fixed nitrogen in the form of ammonia (at least some other proteins that are associated with nitrogen metabolism in this organism, e.g., glutamine synthetase and glutamate synthase, appear to be constitutively synthesized [10]). Synthesis of all five identified *nif* gene products was repressed by atmospheric concentrations of O_2 (Fig. 1e), at least partially explaining some of the difficulties in obtaining nitrogen fixation under dark aerobic conditions. *R. capsulata* has been shown to fix nitrogen under microaerobiosis (12, 18), but at present the permissive oxygen tension is unknown.

Regulatory effects of molybdenum, tungsten, and manganese. The regulatory effects of molybdenum and tungsten appear to vary from organism to organism. In *Klebsiella*, although molybdenum itself has no regulatory properties, it indirectly regulates the synthesis of the nitrogenase subunits, probably by rendering the Mo-Fe protein more stable (5). Although *Clostridium* appears to be similar to *Klebsiella* in terms of regulation of nitrogenase synthesis by molybdenum, in *Azotobacter*, synthesis of the Fe protein shows no metal requirement, and the Mo-Fe protein is synthesized in the presence of either molybdenum or tungsten (3, 14). The cyanobacteria show no metal requirement for full expression of either nitrogenase component (6). Two-dimensional gels of *R. capsulata* show that other *nif* genes, in addition to the *nif* structural genes, are repressed under conditions of molybdenum deprivation, and this effect is not relieved by tungsten.

For studies of the effects of molybdenum or manganese deprivation, the inoculum was from a culture that had been subcultured at least three times on deficient medium. Deficient media were made with doubly distilled water and stock solutions which lacked the appropriate element and which had been treated with Chelex-100 (Bio-Rad) to remove trace impurities. These procedures removed sufficient manganese so that cultures were unable to grow on N_2 (20). When *R. capsulata* cultures were deprived of molybdenum and grown on limiting ammonium, conditions under which normal cultures produce N_2ase A (4), in vivo nitrogenase activity was very low, only about 2% of normal. (Essentially the same results were obtained with lactate-, glutamate-, Mo-deficient cultures). A two-dimensional gel of this culture revealed an interesting pattern of partial repression of *nif* gene products (Fig. 1f). *Nif* proteins 59.5, 55.0 (Mo-Fe protein subunits), and 38.4 kilodaltons appear to be repressed. It is possible that the 38.4-kilodalton protein is involved in Fe-Mo-Co synthesis, or processing and is repressed along with the Mo-Fe protein. The spot corresponding to the Fe protein (33.5 kilodaltons) was also absent, but on some gels a new spot of approximately the same isoelectric point but lower molecular weight was observed. Thus, if the Fe protein is synthesized, it is in an altered or unstable form. It is interesting to note that an apparently constitutively synthesized protein was missing under Mo-deficient conditions (Fig. 1f). The function of this protein is presently unknown. Recently, a novel alternative nitrogen fixation system has been described for *Azotobacter vinelandii* (1). This system, consisting of at least four new proteins that are ammonia repressible, is expressed in molybdenum-starved wild-type cells grown under N_2-fixing conditions. Apparently, this system is not operative in *R. capsulata* since we have failed to detect the described proteins on gels of cells grown in the absence of Mo.

Attempts were made to show preferential synthesis of some or all *nif* gene products upon addition of molybdenum to Mo-deficient cultures. Resting cells (13) were prepared from lactate (30 mM), ammonium (7 mM), and Mo-deficient cultures grown photosynthetically and were incubated under illumination in argon-flushed vials in a Warburg apparatus at 30°C. Cultures were incubated in mineral salt medium (8) supplemented with 50 μCi of [^{14}C]lactate (obtained from International Chemical) with and without Mo (1.2 μM) for 4 h and then assayed for in vivo N_2ase activity. ^{14}C was used (instead of ^{35}S) to monitor the possible synthesis of all proteins modulated by the availability of Mo, not just those containing iron-sulfur groups. A 4-h incubation time was chosen to give sufficient time for substantial protein synthesis to occur under resting cell conditions (no added nitrogen source). Cultures given Mo showed a 25-fold increase in nitrogenase activity over the culture that did not receive Mo (8.1 nmol of C_2H_4 min^{-1} mg of protein^{-1} versus 0.29 nmol of C_2H_4 min^{-1} mg of protein^{-1}). This culture accumulated 2.3 times more radioactive label than did the Mo-deprived culture. However, the majority of the label was found in a 24-kilodalton protein of unknown function. This may correspond to a molybdenum storage or molybdenum transport protein and had the same molecular weight as one of the subunits of the Mo storage protein of *A. vinelandii* (16), but further work is needed to establish this point.

The two-dimensional pattern obtained with Mo-deficient cultures to which tungsten had been added (to a final concentration of 20 μM) was essentially the same as that with Mo-deficient cultures (data not shown). Thus, in *R. capsulata*, tungsten cannot substitute for Mo in promoting nitrogenase synthesis. Manganese has been shown to be required for the diazotropic growth of some photosynthetic bacteria, although it does not appear to be required for nitrogenase activity under nitrogen starvation conditions (20). When two-dimensional gels of manganese-deficient cultures grown on lactate-glutamate were examined, no differences in the *nif* gene products were seen from gels prepared from manganese-sufficient cultures (data not shown).

In conclusion, in this study, the three gene products for the structural proteins of *R. capsulata* nitrogenase were positively identified (by electrophoresis of the purified components), and two other soluble *nif* gene products were tentatively identified, using two-dimensional gel electrophoresis. This method might also prove useful in the study of the general regulation of protein expression under the five different growth modes available to *R. capsulata*. In three different types of cultures, with ammonia, oxygen, or with the Nif$^-$ mutant W15, the five putative *nif* gene products were not expressed. Four of the five were also not expressed under molybdenum deprivation. This strongly suggests that all five proteins are *nif* gene products; conclusive demonstration of *nif* gene products will require the isolation and characterization of Nif$^-$ mutants (an ongoing area of active research in this laboratory).

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ADDENDUM IN PROOF

Recent results indicate that protein number four (55 kilodaltons; pI = 5.65) may be glutamine synthetase (W. P. Michalski, P. M. Vignais, and D. J. D. Nicholas, abstr. no. 295, Abstract Book, IUB-IUPAB Bioenergetics Group, Second European Bioenergetics Conference, Lyon, July 1982).

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