Inhibition of Hydrogenase Synthesis by DNA Gyrase Inhibitors in *Bradyrhizobium japonicum*†

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Derepression of an uptake hydrogenase in *Bradyrhizobium japonicum* is dependent on a microaerophilic environment. Addition of DNA gyrase inhibitors during derepression of hydrogenase specifically prevented expression of the hydrogenase enzyme. Antibodies to individual hydrogenase subunits failed to detect the protein after derepression in the presence of inhibitors, although there was no general inhibition of protein synthesis. The general pattern of proteins synthesized from 14C-labeled amino acids during derepression was not significantly different whether proteins were labeled in the presence or in the absence of gyrase inhibitors. In contrast, if transcription or translation was inhibited by addition of inhibitors of those functions, virtually no proteins were labeled during derepression. This indicated that most of the 14C-labeled proteins were synthesized de novo during derepression, synthesis of most proteins was unaffected by gyrase inhibitors, and the dependence of hydrogenase synthesis on gyrase activity was a specific one.

There is a correlation between the expression of enzymes which control the tertiary structure of DNA and gene expression in bacteria (reviewed in references 4 and 16). Two enzymes which are responsible for controlling the degree of superhelicity of DNA are DNA gyrase and topoisomerase I. Early work with defined templates showed differences in the level of gene expression depending on the degree of supercoiling of the template DNA (3, 17, 18). These studies link supercoiling, transcription, and gyrase and topoisomerase activities in a general way.

Gyrase inhibitors were known to inhibit transcription of specific genes even before the link between these drugs and gyrase activity was established (20). Numerous studies (6, 8, 19–22, 27, 29) have shown that a variety of genes are transcribed at altered rates in the presence of gyrase inhibitors. While these studies demonstrate that various genes depend on gyrase activity for expression, it is not clear why it is these particular genes which are so affected. Many of the genes which seem to depend on gyrase activity belong to catabolite-sensitive operons (19, 20, 22), but the data indicate that this characteristic alone is not the determining factor.

More recently, several groups have demonstrated a relationship between DNA supercoiling and activation of a group of genes, all of which are controlled by oxygen. Mutants of the facultative anaerobe *Salmonella typhimurium* which cannot grow anaerobically have been isolated and shown to lack gyrase activity. Conversely, mutants which cannot grow aerobically have been shown to lack topoisomerase I activity (28). Looking at a more specific set of genes, Kranz and Haselkorn have shown that anaerobic induction of the nitrogen fixation genes in *Rhodopseudomonas capsulata* or *Klebsiella pneumoniae* can be prevented by including gyrase inhibitors under normally derepressing conditions (7). Other proteins which are induced anaerobically, such as dimethyl sulfoxide reductase, photosynthesis-related proteins, and those involved generally in anaerobic growth functions, are also specifically inhibited by the gyrase inhibitor novobiocin (7).

In this work, *Bradyrhizobium japonicum* was derepressed for expression of the enzyme hydrogenase in the presence of gyrase inhibitors. 14C-labeled amino acids were used to label specifically those proteins made during derepression, and hydrogenase was detected antigenically. Although most of the proteins made during derepression were unaffected, hydrogenase was not detected in the presence of these inhibitors; its synthesis depended on gyrase activity.

**MATERIALS AND METHODS**

**Chemicals.** Novobiocin, nalidixic acid, and rifampin were obtained from Sigma Chemical Co., St. Louis, Mo., and chloramphenicol was from Boehringer Mannheim Biochemicals, Indianapolis, Ind. Gases were obtained from Arundel Sales and Service, Baltimore, Md. 14C-labeled amino acids, with a specific activity of 10 mCi/mmol, were purchased from New England Nuclear Corp., Boston, Mass. Coumermycin was a gift from Bristol-Myers, Pharmaceutical Research Div., Wallingford, Conn. Coumermycin, insoluble in water, was dissolved in dimethyl sulfoxide before use. Nickel chloride was Puratronic grade, from Johnson Mathey Chemicals Ltd., Hartfordshire, England. All other chemicals were reagent grade.

**Media and cell growth.** *B. japonicum* SR (12, 15) was grown on modified Bergersen medium (MB) (1) to a cell density of 3 \( \times 10^8 \) cells per ml. Cells were derepressed for hydrogenase activity in no-carbon medium (12) at a density of 2 \( \times 10^8 \) cells per ml. NiCl\(_2\) was routinely added to both MB and no-carbon media to a concentration of 5 \( \mu \)M.

**Derepression of hydrogenase.** At a cell density of 3 \( \times 10^8 \) cells per ml, bacteria were harvested, washed once, and suspended in no-carbon medium (12). Inhibitors of gyrase, transcription, or translation were added at the various concentrations indicated in the figure legends. When used, the gyrase inhibitors novobiocin, coumermycin, and nalidixic acid were added individually at concentrations ranging from 5 to 500 \( \mu \)g/ml. Similarly, when used, rifampin and chloramphenicol were added, alone or in conjunction with one of the gyrase inhibitors, at concentrations ranging from 25 to 100 \( \mu \)g/ml. When cells were to be labeled, 2 \( \mu \)Ci (per 10 ml of
cells) of $^{14}$C-labeled amino acids was added with the inhibitor(s) at the start of derepression. Samples (10 ml) of cells were derepressed for 14 or 16 h under 0.5% oxygen as described previously (14, 24). Hydrogenase activity was assayed amperometrically as reported earlier (5, 26).

**Cell viability.** Cells were assayed for viability by serially diluting them in 0.9% saline and then plating them on MSY medium (11).

**Gel electrophoresis.** The discontinuous polyacrylamide gel electrophoresis system described by Laemmli (9) was used, and the resolving gel was 10% acrylamide. The 10-ml samples of derepressed cells were harvested, washed once in 50 mM potassium phosphate buffer (pH 7.0), and suspended vigorously in 100 µl of sodium dodecyl sulfate (SDS) sample buffer (9). Samples were alternately boiled and quick-frozen in dry ice-ethanol through four successive cycles. Frozen samples were stored in the dry ice-ethanol bath or at −20°C until needed and boiled again immediately before use. The samples were centrifuged briefly to pellet cell debris, and 10 µl of each resultant supernatant was loaded per gel lane. Gels were run at 10 mA and run overnight until the bromphenol blue tracking dye left the gel. After electrophoresis, the duplicate thirds of the gels were cut apart and used to detect total protein, labeled proteins, or hydrogenase. After electrophoresis, gel portions were prepared for fluorography essentially as described by Bonner and Laskey (2). Gels were exposed to Kodak XAR-5 film at ~70°C overnight.

**Immunoblotting and antigen detection.** After electrophoresis, gel portions were transferred onto nitrocellulose (0.22-µm pore size; Schleicher & Schuell, Inc., Keene, N.H.) for a Western blot by the method of Towbin et al. (25). The blots were blocked with BLOTTO and then incubated with antibody to the 65- or 33-kilodalton (kDa) subunit of hydrogenase (23). Antigen detection was basically by the method of Stutls et al. (23) with the following modifications. Incubation with the antibody to hydrogenase was overnight in the presence of trace amounts of azide. Incubation with the secondary antibody was also overnight, but in the presence of trace amounts of thimerosal.

**RESULTS AND DISCUSSION**

**Inhibition of hydrogenase activity.** *B. japonicum* can be derepressed for hydrogenase activity if it is placed in an environment which lacks carbon substrates and which is also microaerophilic (10, 13). Since hydrogenase is negatively regulated by oxygen, it was potentially a member of the class of proteins whose synthesis depends on gyrase activity. To test this idea, cells were derepressed for hydrogenase under non-growing conditions in the presence of gyrase inhibitors, and the resulting levels of hydrogenase activity were measured. The effects of the inhibitors on activity are summarized in Table 1. The wild-type activity (100%) was 88 nmol/h per 10⁸ cells. All three gyrase inhibitors strongly inhibited derepression of hydrogenase activity in a 14-h period. Even 5 µg of novobiocin or coumermycin per ml significantly inhibited expression of hydrogenase activity, and either drug at 10 µg/ml was sufficient to eliminate at least 90% of the activity relative to that of control cells. Nalidixic acid proved less potent, but at 50 µg/ml it inhibited derepression to 61% of the level of control cells. At the end of the derepression period, the optical densities of inhibited cells were similar to those of control cells derepressed in the absence of inhibitors, indicating that the inhibitors did not cause cell death and subsequent lysis. To test this, however, cells were plated for viability. Cell viability was slightly decreased in the presence of 200 µg of novobiocin per ml (80% of control), but 100 µg of that drug per ml, a concentration sufficient to eliminate hydrogenase activity, had no effect on viability.

Although coumermycin and novobiocin were potent inhibitors of hydrogenase derepression, they did not affect hydrogenase activity if they were added to previously derepressed cells. At 100 µg/ml, neither novobiocin nor coumermycin had any effect on the activity of cells which had already synthesized hydrogenase protein.

**Protein synthesis.** To examine the effects of the inhibitors on protein synthesis, cells were labeled with a $^{14}$C-amino acid mixture during derepression. A 2-µCi (per 10 ml of cells) portion of labeled amino acids was added with the inhibitors at the start of derepression. At the end of the derepression period, the samples were broken in SDS sample buffer and electrophoresed on polyacrylamide gels. The gels were run as identical thirds so that total proteins, labeled proteins, and hydrogenase protein could be examined. Silver staining (data not shown) revealed that the patterns of most proteins were not altered by addition of gyrase inhibitors during derepression. In addition, cells derepressed in the presence of inhibitors contained the same amount of incorporated radioactivity per cell as did control cells derepressed without inhibitor. This indicated that normal levels of protein synthesis occurred even in the presence of inhibitors.

More importantly, fluorograms clearly revealed the profiles of those proteins made specifically in the presence of the inhibitors (Fig. 1A). Although the levels of inhibitors used for this experiment were in excess of the concentrations necessary to inhibit hydrogenase activity completely, there were few differences in the general patterns of newly syn-

| TABLE 1. Effects of gyrase inhibitors on derepression of hydrogenase activity |
|-----------------------------|------------------|
| Derepression condition | % Hydrogenase activity* |
|-------------------------|-------------------|
| Dimethyl sulfoxide (0.1 ml) | 97 |
| Coumermycin (µg/ml) | |
| 5                       | 20               |
| 10                      | 6                |
| 50                      | <1               |
| 100                     | <1               |
| Novobiocin (µg/ml)      | |
| 5                       | 35               |
| 10                      | 10               |
| 50                      | <2               |
| 100                     | <1               |
| 200                     | <1               |
| 500                     | 0                |
| Nalidixic acid (µg/ml)  | |
| 5                       | 90               |
| 10                      | 84               |
| 50                      | 61               |
| 100                     | 39               |
| 200                     | 16               |
| 250                     | <2               |

* Hydrogenase activities were measured amperometrically after 14 h of derepression in the presence of the inhibitor listed. Activities are expressed as percentages of the control, which was the activity of wild-type cells derepressed in the absence of inhibitor. The activity of the control was 88 nmol/h per 10⁸ cells.
thesized proteins. Most of the differences which did exist were quantitative rather than qualitative. The reaction catalyzed by hydrogenase can serve as a source of energy, so at least some of the differences in the protein patterns could be a result of the lack of hydrogenase activity, rather than being caused directly by the gyrase inhibitors.

**Hydrogenase synthesis.** In contrast to their effects on total protein synthesis, gyrase inhibitors did affect the synthesis of hydrogenase protein. Western blots (Fig. 1B) showed that the 65-kDa subunit of hydrogenase was not synthesized when inhibitors were present during derepression. There was an obvious antibody-specific reaction in lanes containing samples from cells derepressed without inhibitors (lanes 1 and 2), but cells incubated in the presence of coumermycin or novobiocin contained no material cross-reactive with hydrogenase. Western blots with antibody to the 33-kDa subunit of hydrogenase as the primary antibody yielded the same results (Fig. 2). In contrast, Western blots of samples in which hydrogenase had been only partially inhibited showed a small amount of cross-reactive material (data not shown). These results agreed with the data obtained from amperometric assays of hydrogenase activity. They showed that although most of the proteins labeled during derepression were unaffected by addition of gyrase inhibitors, hydrogenase was not synthesized in the presence of those inhibitors.

**Inhibition by rifampin or chloramphenicol.** It was possible that the gyrase inhibitors inhibited transcription or translation in a nonspecific way, and the $^{14}$C-labeled protein patterns of Fig. 1 resulted from labeling preexisting, stable messages or polypeptides that existed before derepression.

**FIG. 1.** SDS-polyacrylamide gel of cell extracts after derepression in the presence of gyrase inhibitors. Samples (10 μl) containing approximately 60,000 cpm (slightly less in lane 4) were loaded in each lane. Panels A and B show duplicate halves of the same gel. Cells were derepressed in the presence of the following (lanes): 1, no inhibitor (control cells); 2, 0.1 ml of dimethyl sulfoxide; 3, 50 μg of coumermycin per ml; 4, 100 μg of coumermycin per ml; 5, 100 μg of novobiocin per ml; 6, 200 μg of novobiocin per ml. (A) Fluorogram of proteins labeled with $^{14}$C-amino acids during derepression. (B) Western blot with affinity-purified antibody to the 65-kDa subunit of hydrogenase.

**FIG. 2.** Western blot of a polyacrylamide gel containing the same samples as those in Fig. 1, probed with affinity-purified antibody to the 33-kDa subunit of hydrogenase. Cells were derepressed in the presence of the following (lanes): 1, 200 μg of novobiocin per ml; 2, 100 μg of novobiocin per ml; 3, 100 μg of coumermycin per ml; 4, 50 μg of coumermycin per ml; 5, no inhibitor.

Cells were examined for hydrogenase activity, therefore, in the presence of known inhibitors of transcription and translation. Cells were derepressed for hydrogenase activity in the presence of either rifampin, an inhibitor of transcription, or chloramphenicol, an inhibitor of translation. The inhibitors were added at the start of the derepression period. After 16 h, hydrogenase activity was measured amperometrically. Table 2 shows that addition of either drug prevented derepression of hydrogenase activity. In the presence of 25 μg of rifampin or chloramphenicol per ml, cells expressed 6% or less of the level of hydrogenase activity expressed by control cells. Thus, hydrogenase activity during derepression depends on mRNA and protein synthesis.

**Specificity of label.** It was important to determine whether

**TABLE 2.** Effects of chloramphenicol and rifampin on derepression of hydrogenase activity

| Inhibitor and concn (μg/ml) | % Hydrogenase activity<sup>a</sup> |
|----------------------------|-----------------------------------|
| Chloramphenicol            |                                    |
| 25                         | <1                                 |
| 50                         | <1                                 |
| 100                        | <1                                 |
| Rifampin                   |                                    |
| 25                         | 6                                  |
| 50                         | 5                                  |
| 100                        | 3                                  |
| 200                        | <1                                 |

<sup>a</sup> Hydrogenase activities were assayed amperometrically after 16 h of derepression in the presence of the inhibitor shown. Activities are expressed as percentages of the control, which was the activity of wild-type cells derepressed without inhibitors. The activity of the control cells was 225 nmol/h per 10<sup>8</sup> cells.
cells derepressed in the presence of novobiocin or coumermycin showed patterns of labeled proteins very similar to those of control cells, and on a per-cell basis the cells incubated with gyrase inhibitors contained the same amount of incorporated radioactivity as did control cells. When rifampin or chloramphenicol was added during derepression, either alone or in combination with one of the gyrase inhibitors, there was little or no labeling of cells by the \(^{14}C\)-amino acids (lanes 3, 4, and 5). Since general inhibition of transcription or translation eliminated labeling, the \(^{14}C\)-amino acids were incorporated into proteins transcribed and translated de novo during derepression; previously existing messages or proteins were not substrates for labeling. Gyrase inhibitors, then, did not alter overall transcription, and their effect on hydrogenase synthesis was a specific one. Although both de novo transcription and translation occurred normally during derepression in the presence of gyrase inhibitors, hydrogenase was not synthesized.

Thus, derepression of hydrogenase in *B. japonicum* can be prevented by drugs known to inhibit gyrase activity. Hydrogenase, is regulated in some way by the level of oxygen in the environment. Some common regulatory element must affect the variety of proteins present in this or other bacteria which are controlled by oxygen tension. Gyrase, as an enzyme which controls the tertiary structure of DNA, would be a good candidate for such a regulator. In addition, there is precedence for a link between inhibition of gyrase activity and expression of anaerobic proteins (7). There is also a direct correlation between gyrase activity and anaerobic
gene expression in bacteria (28). We have demonstrated specific inhibition of hydrogenase synthesis when gyrase inhibitors were added to derepressing cells. Thus, gyrase activity, and perhaps extent of DNA supercoiling, seems to be an important element in the conditions essential to the synthesis of hydrogenase in *B. japonicum*.

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