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Transcription Termination Factor Rho Is Essential for *Micrococcus luteus*

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The growth of *Micrococcus luteus*, a soil microorganism that belongs to the high-G+C gram-positive phylogenetic group, is prevented by bicyclomycin, an antibiotic that inhibits the activity of the *M. luteus* transcription termination factor Rho. A mutant that can grow in 0.3 mM bicyclomycin has a Rho that is insensitive to bicyclomycin and has the single amino acid residue change of Asp^{474} to Gly. These results indicate that the function of its Rho factor is essential for *M. luteus* and that growth of a gram-positive organism can be blocked by bicyclomycin.

Very little is known about the mechanisms for termination of transcription in bacterial organisms other than *Escherichia coli*. In particular we do not know whether all organisms are like *E. coli* in having two major mechanisms, one that is intrinsic to the function of RNA polymerase itself and the other dependent on the action of a protein factor called Rho. *Bacillis subtilis* RNA polymerase is known to terminate transcription spontaneously at sequences that closely resemble intrinsic terminators from *E. coli* (1). Since *B. subtilis* is not closely related to *E. coli*, this result suggests that the intrinsic termination mechanism is likely conserved in the *Bacteria*. In addition, the genes encoding the subunits of RNA polymerase core from a wide variety of organisms have strongly conserved features (12, 13). From this observation we infer that the enzymes in all *Bacteria* function by the same basic mechanism, a mechanism that would include release of transcripts at intrinsic terminators. As to the prevalence of the Rho-dependent mechanism, genes encoding proteins that would be very similar in structure to *E. coli* Rho have been found in a variety of highly divergent organisms (10), suggesting that the termination function of Rho is highly conserved. However, a *rho* homolog is not present in the minimal genome of *Mycoplasma genitalium* (2), a parasitic pathogen, and functional Rho factors have been isolated from very few organisms.

In *E. coli*, *rho* is an essential gene. Since efficient termination of transcription at the ends of some operons is known to be dependent on the function of Rho-dependent terminators (11, 14), a likely explanation for the loss of viability in the absence of Rho is that inadvertent transcription of certain sequences of DNA occurs, causing the production of toxic substances. Another possibility is that the cumulative consequences of the inappropriate expression of genes and the synthesis of antisense RNA due to the absence of Rho create a catastrophic situation for the cell.

If the efficient function of Rho-dependent terminators is as important in other bacteria as it is in *E. coli*, a blockage of Rho function in those cells should cause loss of viability. Recently, we isolated Rho factor from *Micrococcus luteus* (8), an organism that is a member of the high-G+C gram-positive phylogenetic group and thus is only distantly related to *E. coli*. We showed that *M. luteus* Rho does function as a transcription terminator factor in vitro. We also found that it is inhibited by bicyclomycin (8), an antibiotic that is known to be effective against several gram-negative bacteria but did not block the growth of some gram-positive bacteria, including *B. subtilis* and *Staphylococcus aureus* (6). In *E. coli* the target for the toxic effect of bicyclomycin is Rho factor. This was shown by the isolation of bicyclomycin-resistant mutants of *E. coli* that have changes in the *rho* gene and that have bicyclomycin-resistant Rho factors (15). The finding that *M. luteus* Rho is inhibited by low concentrations of bicyclomycin [0.2 mM bicyclomycin inhibits poly(C)-dependent ATPase and transcription termination activities by more than 95% (8)] raised the possibility that this gram-positive organism might be sensitive to this antibiotic. Such a sensitivity would then provide a means for demonstrating whether Rho is essential for the growth of this organism.

***M. luteus* is sensitive to bicyclomycin.** When 1.6×10^2 *M. luteus* cells were spread on agar plates containing 90 μg of bicyclomycin/ml (0.3 mM) in 2× YT medium (16 g of Bacto Tryptone [Difco], 10 g of yeast extract [Difco], and 5 g of NaCl per liter of H_2O), no colonies appeared after 2 days. When this number of cells was spread on plates containing 30 and 60 μg of bicyclomycin/ml, respectively, 80 ± 8 and 40 ± 4 very minute colonies appeared after a day. Hence, *M. luteus* is sensitive to bicyclomycin at a 50% inhibitory concentration of 30 μg/ml, which is very similar to the value determined for *E. coli* W3350 (15). This is the first example of a gram-positive organism that is sensitive to bicyclomycin.

To isolate mutants that are resistant to the antibiotic, we used UV to induce mutations. *M. luteus* cells growing exponentially in a broth culture were pelleted, washed, and suspended in 0.1 M $MgSO_4$ at a density of $\sim 5 \times 10^8$ cells/ml. A 5-ml suspension was exposed for 3 min at a distance of 30 cm to two germicidal lamps, yielding a survival rate of 22% and eight colonies that grew on 2× YT plates containing 90 μg of bicyclomycin/ml. One clone, Mlu-A, was selected for further analysis. Mlu-A cells showed viabilities of 100 and 70% on plates with 75 and 300 μg of bicyclomycin/ml, respectively. Thus the 50% inhibitory concentration of bicyclomycin for Mlu-A was more than 10-fold greater than that for wild-type *M. luteus*. Its growth rate (data not shown) and growth yields (Fig. 1) in liquid culture were unaffected by bicyclomycin at concentrations up to 1 mM.

Mlu-A Rho is less sensitive to bicyclomycin than is wild-type *M. luteus* Rho. To test whether Rho from Mlu-A is resistant to

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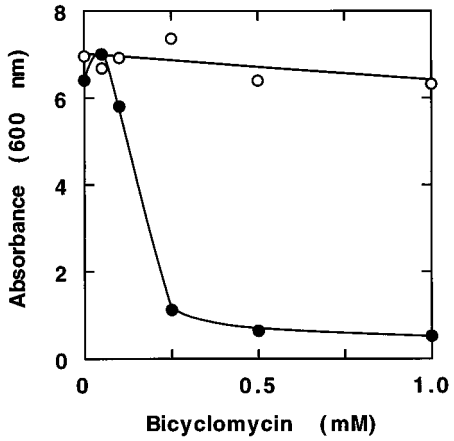

FIG. 1. Comparison of growth yields in liquid cultures for wild-type and Mlu-A mutant strains of *M. luteus* as a function of bicyclomycin concentration. Growth yields for wild-type (●) and Mlu-A (○) strains were measured by the turbidity (optical absorbance) of cultures at 600 nm after incubation with shaking for 22 h at 37°C in 2× YT containing the indicated amounts of bicyclomycin. All cultures were within 90% of the saturation point after 10 h at 37°C.

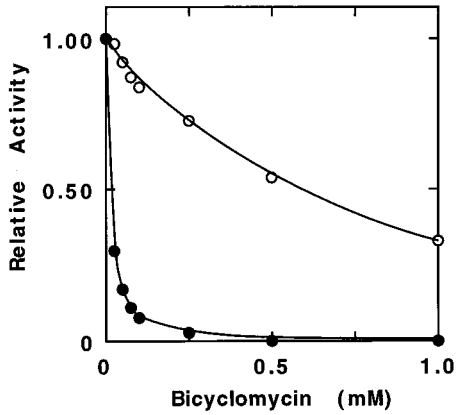

FIG. 3. Effects of bicyclomycin on poly(C)-ATPase activity of purified wild-type and Mlu-A Rho proteins of *M. luteus*. The poly(C)-dependent ATPase activities of 0.15 and 0.25 μg of partially purified wild-type (●) and Mlu-A (○) Rho proteins, respectively, were assayed as described previously (7) in the presence of the indicated concentrations of bicyclomycin. The amounts of ATP hydrolyzed in 10 min in the absence of bicyclomycin were 4.3 and 4.2 nmol for wild-type and Mlu-A Rhos, respectively.

bicyclomycin, the Rho proteins were purified from cultures of the wild-type and Mlu-A mutant strains by chromatography on Bio-Rex 70 and were concentrated by centrifugation with a Microcon-50 filter. This purification was sufficient to remove contaminating ATPases and yielded preparations that were ~25% pure for wild-type Rho and >75% pure for the mutant Rho (Fig. 2). These partially purified proteins were then tested for inhibition of their poly(C)-dependent ATPase activities with bicyclomycin.

The results indicate that the Mlu-A Rho protein was approximately 10-fold less sensitive to bicyclomycin than the wild-type protein (Fig. 3). At a bicyclomycin concentration of 0.25 mM (75 μg/ml) the ATPase activity of wild-type Rho was nearly completely abolished, whereas Mlu-A Rho remained 75% active. At the highest concentration of bicyclomycin tested (1 mM), the mutant Rho protein retained approximately 30% of its poly(C)-dependent ATPase activity.

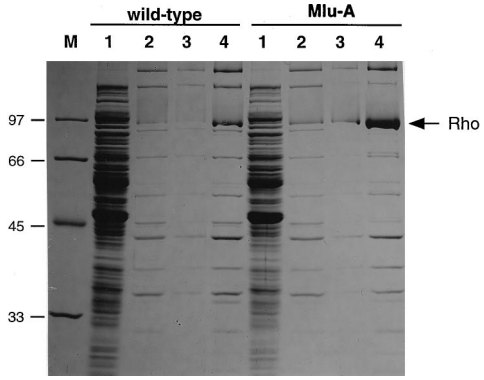

FIG. 2. Gel electrophoresis analysis comparing the effectiveness of purification of the Rho proteins. *M. luteus* wild-type and Mlu-A Rho proteins were partially purified from 1 g (wet weight) of cells as previously described (9) by Bio-Rex 70 chromatography and were concentrated by centrifugation through a Microcon-50 filter (Amicon). The samples were separated by electrophoresis on a 10% polyacrylamide gel with the Laemmli buffer system (4). Lane M, marker proteins (sizes in kilodaltons); lanes 1, cell lysates; lanes 2, Bio-Rex 70 pools; lanes 3 and 4, 3 and 12 μg, respectively, of Microcon-50 retentates.

Identification of the change in the DNA sequence. To determine the change in Rho protein responsible for the resistance, the 3-kb *Bam*HI-*Sac*I fragments of Mlu-A genomic DNA were ligated into a Bluescript vector and a clone containing the plasmid with the correct insert was detected by using a colony DNA hybridization protocol with a labeled *Mlu rho* DNA as a probe (8). The DNA sequence of the open reading frame in the insert in the plasmid in that clone was determined in its entirety. A single nucleotide change was located within the codon for residue 474, changing it from GAC to GGC (D474G). Asp^{474} is in the ATP-binding domain (8, 10), and the corresponding residue is absolutely conserved in all known sequences of putative Rho factor (10), implicating it in a crucial functional role. The corresponding residue in *E. coli* Rho is Asp^{210}, which is located on a loop between β strand 4 and α helix C in the tertiary structure model for the ATP-binding domain proposed by Miwa et al. (5). This residue is close to Met^{219}, which is changed to Lys in one of the bicyclomycin-resistant mutants of *E. coli* Rho isolated by Zwiefka et al. (15). Since Met^{219} is also in α helix C of the Miwa et al. model, bicyclomycin may make contact with that α helix.

In the course of checking the sequence of the cloned DNA for all possible changes, we found that a mistake had been made in the original sequence reported for the *M. luteus rho* gene; the A and G residues in the codon for amino acid 501 had been transposed. The corrected sequence is GAC, not AGC, and encodes an Asp residue, not a Ser. (The sequence for *M. luteus rho* deposited with GenBank, accession no. L27277, has been corrected.) The sequences of all the other *rho* genes from the high-G+C gram-positive phylogenetic group also have Asp at that position. Therefore, the sole mutation in *rho* of *M. luteus* Mlu-A which makes the cells less susceptible to inhibition by bicyclomycin is D474G.

We have shown for the first time that transcription termination factor Rho is essential for the viability of a gram-positive bacterium and also that a gram-positive organism is sensitive to the antibiotic bicyclomycin. Recently Ingham et al. (3) demonstrated that the Rho factor from *Streptomyces lividans*, another organism in the high-G+C gram-positive group, was inhibited by bicyclomycin in vitro but that growth of the organism was unaffected by this antibiotic. In view of its close phylogenetic relatedness to *M. luteus*, we believe that Rho is

equally likely to be essential in *S. lividans*. The resistance of *S. lividans* to bicyclomycin could be due to an inability of the compound to enter the cells. Included in the same phylogenetic group is the important pathogen *Mycobacterium tuberculosis*. Although its growth was not blocked by bicyclomycin (6), we strongly suspect that its Rho factor is essential for viability and will be inhibited by bicyclomycin in vitro. These assumptions should present a sufficiently strong incentive to develop a derivative of bicyclomycin that would be effective in combating tuberculosis.

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