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Increased Levels of Dihydrofolate Reductase in Rifampin-Resistant Mutants of *Bacillus subtilis*

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Several independent, spontaneous rifampin-resistant mutants of *Bacillus subtilis* were isolated and found to have an increased resistance to trimethoprim, an inhibitor of dihydrofolate reductase. This increased resistance in the *rif* mutants was the result of a specific threefold increase in the activity of dihydrofolate reductase, since six other enzymes examined remained unchanged. This increased level of dihydrofolate reductase and the trimethoprim resistance were cotransformed (100%) with the *rif* marker. These results suggest that the RNA polymerase is altered in its recognition of the gene that specifies dihydrofolate reductase.

Rifampin and its derivatives are antibiotics that inhibit the activity of the DNA-dependent RNA polymerase by binding to the β-subunit of the enzyme and preventing the initation of transcription (2, 7). Mutations to rifampin resistance have been reported to have pleiotropic effects on complex metabolic processes, such as: a reduced ability of *Bacillus subtilis* to sporulate (6, 12, 13); suppression of *dnaA* mutations (1); and inhibition of the growth of bacteriophages (11). In addition, alterations in specific enzymes have been reported in some *rif* mutants; specifically, an increased level of enzymes in the arginine pathway (17), an increased level of dihydrofolate (DHF) reductase (M. M. Aldridge, V. J. Wainscott, and J. F. Kane, Abstr. Annu. Meet. Am. Soc. Microbiol. 1976, K47, p. 144), and the absence of the enzyme glutamate synthase (10). In this report we present results that show an increased level of DHF reductase in *rif* mutants of *B. subtilis*.

All of the mutants used in this study were derivatives of the competent strain 168. Spontaneous rifampin-resistant mutants were obtained by surface spreading the *gat-7* mutant (previously referred to as the *trpX7* mutant [4, 5]) NP102 on Trypticase soy agar containing 0.6% yeast extract and 5 μg of rifampin (Sigma Chemical Co., St. Louis, Mo.) per ml. The plates were incubated at 37°C until resistant colonies appeared. Single colonies were transferred to the rifampin-containing medium and streaked for isolation. Cultures for enzyme assays were routinely grown in 200 ml of minimal glucose medium at 37°C, and crude extracts were prepared as previously described (4). DHF reductase was measured at 37°C in a 1-ml reaction mixture containing 40 mM potassium phosphate (pH 7.5), 0.1 mM NADPH, and 0.05 mM dihydrofolic acid (Sigma Chemical Co.). The following enzymes were measured as previously described: XMP amidotransferase (9), acetylornithine δ-transaminase (15), glutamate synthase (8), serine transhydroxymethylase (14), anthranilate synthase (4), and *p*-aminobenzoate (PABA) synthase (5). DNA was isolated and transformation experiments were carried out as previously described (3).

The *gat-7* mutant NP102 has a defect in the small protein subunit G (previously referred to as subunit X [4]) of the enzyme PABA synthase (5) and as a result has a low pool level of PABA, an essential moiety of DHF. A further consequence of this mutation is seen in Table 1. The mutant NP102 was hypersensitive to inhibition by trimethoprim, a drug that is a competitive inhibitor of DHF reductase with respect to DHF. This increased sensitivity was alleviated by supplementing the medium with PABA (Table 1). Since comparable supplements of PABA did not influence the trimethoprim inhibition of the prototroph, NP1, we conclude that the mutant NP102 has a low pool level of both PABA and DHF.

Spontaneous rifampin-resistant derivatives of mutant NP102 were isolated. Of 50 mutants examined, 10 had an increased resistance to trimethoprim (Table 2) but possessed both the original *gat* mutation and the hypersensitivity to sulfathiazole of the parental mutant NP102. The growth rates of the prototroph were also included for comparison. DHF reductase from

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TABLE 1. *Effect of trimethoprim and PABA on the growth of B. subtilis*[a]

Concentration of trimethoprim (μg/ml)	Specific growth rate (*k*)			
	Prototroph		*gat-7* Mutant	
	−PABA	+PABA	−PABA	+PABA
0	0.54	0.54	0.53	0.55
0.03	—	—	0.39	0.52
0.05	—	—	0.21	0.55
0.10	0.52	0.50	0.07	0.50
0.20	0.45	0.46	0	0.49
0.30	0.39	0.40	0	0.32

[a] Cells were grown in 8 ml of minimal glucose medium containing the indicated concentrations of trimethoprim. PABA was added to a final concentration of 100 ng/ml where indicated. Growth rates (*k*) were determined during the first 10 h after inoculation from overnight cultures.

TABLE 2. *Growth patterns of the rif gat-7 mutants*

Collection no.	Genotype	Specific growth rate (*k*)[a] with supplements:		
		None	Sulfathiazole (0.5 μg/ml)	Trimethoprim (0.1 μg/ml)
NP1	Prototroph	0.53	0.50	0.55
NP102	*gat-7*	0.55	0.07	0.09
GT1	*rif-1 gat-7*	0.54	0.08	0.09
GT4	*rif-4 gat-7*	0.50	0.10	0.43
GT7	*rif-7 gat-7*	0.49	0.09	0.23
GT13	*rif-13 gat-7*	0.53	0.09	0.29

[a] Cultures were grown as described in Table 1. The indicated concentrations of sulfathiazole and trimethoprim are final concentrations in the growth medium.

these *rif* mutants was assayed (Table 3). All *rif* mutants with an increased resistance to trimethoprim had about a threefold increase in the specific activity of DHF reductase.

We then examined the following enzymes to determine the specificity of the effect caused by this *rif* mutation: glutamate synthase, XMP amidotransferase, anthranilate synthase, PABA synthase, serine transhydroxymethylase, and acetylornithine δ-transaminase. All of these enzymes were essentially the same in the *rif* mutant and the parental mutant NP102.

The pleiotropic effect of the *rif* mutation was verified by transformation. The mutation that specifies rifampin resistance was located in the same region (3) as previously described *rif* mutations that result in an altered β-subunit of RNA polymerase. This locus, which codes for the β-subunit of RNA polymerase, is located near the origin of DNA replication. The locus that specifies resistance to trimethoprim is cotransformed with the *metB* locus (16) and is

TABLE 3. *DHF reductase activity of rif gat-7 mutants*

Collection no.	Genotype	Relative activity[a]
NP1	Prototroph	1.0
NP102	*gat-7*	0.9
GT1	*rif-1 gat-7*	1.1
GT4	*rif-4 gat-7*	2.9
GT7	*rif-7 gat-7*	2.6
GT13	*rif-13 gat-7*	3.1

[a] The relative activity of 1 is equal to 34 nmol of DHF reduced per min per mg of protein.

located near the terminus of DNA replication. The prototroph NP1 was transformed to Rif^r with DNA from mutant GT4. All of the 200 transformants examined from such a cross had an increased resistance to trimethoprim. Enzymatic analyses were performed on 15 of these 200 transformants. All 15 mutants had a two- to threefold increase in the specific activity of DHF reductase as compared to the rifampin-sensitive recipient NP1. Since the gene that specifies rifampin resistance is not linked to the gene that specifies trimethoprim resistance, we believe that increased level of DHF reductase, and hence trimethoprim resistance, is a pleiotropic manifestation of the mutation to rifampin resistance.

The effect of the altered RNA polymerase on DHF reductase may have two possible explanations. First, the level of a metabolite essential in the control of this enzyme may be altered. Second, the promotor region of the locus that specifies the enzyme DHF reductase may be read more efficiently in the *rif* mutants than in the parental rifampin-sensitive strain.

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LITERATURE CITED

1. **Bagdasarian, M. M., M. Izakowska, and M. Bagdasarian.** 1977. Suppression of the DnaA phenotype of mutations in the *rpoB* cistron of ribonucleic acid polymerase in *Salmonella typhimurium* and *Escherichia coli*. J. Bacteriol. **130**:577–582.
2. **Hartman, G., K. O. Honikel, F. Knuseh, and J. Nüesch.** 1967. The specific inhibition of the DNA-directed RNA synthesis of rifamycin. Biochim. Biophys. Acta **145**:843–844.
3. **Kane, J. F., R. L. Goode, and J. Wainscott.** 1975. Mutiple mutations in *cysA14* mutants of *Bacillus subtilis*. J. Bacteriol. **121**:204–211.
4. **Kane, J. F., W. M. Holmes, and R. A. Jensen.** 1972. Metabolic interlock. The dual function of a folate pathway gene as an extraoperonic gene of tryptophan biosynthesis. J. Biol. Chem. **247**:1587–1596.
5. **Kane, J. F., and H. D. O'Brien.** 1975. *p*-Aminobenzoate synthase from *Bacillus subtilis*: amidotransferase composed of nonidentical subunits. J. Bacteriol. **123**: 1131–1138.

6. **Leighton, T. J.** 1973. An RNA polymerase mutation causing temperature-sensitive sporulation in *Bacillus subtilis*. Proc. Natl. Acad. Sci. U.S.A. **70:**1179–1183.
7. **Linn, T., R. Losick, and A. L. Sonenshein.** 1975. Rifampin resistance mutation of *Bacillus subtilis* altering the electrophoretic mobility of the beta subunit of ribonucleic acid polymerase. J. Bacteriol. **122:**1387–1390.
8. **Meers, J. L., D. W. Tempest, and C. M. Brown.** 1970. Glutamine (amide): 2-oxoglutarate amino transferase oxidoreductase (NADP), an enzyme involved in the synthesis of glutamate by some bacteria. J. Gen. Microbiol. **64:**187–194.
9. **Patel, N., H. S. Moyed, and J. F. Kane.** 1977. Properties of xanthosine 5′-monophosphate-amidotransferase from *Escherichia coli*. Arch. Biochem. Biophys. **178:** 652–661.
10. **Ryu, J.-I.** 1978. Pleiotropic effect of a rifampin-resistant mutation in *Bacillus subtilis*. J. Bacteriol. **135:**408–414.
11. **Snyder, L. R.** 1972. An RNA polymerase mutant of *Escherichia coli* defective in the T4 viral transcription program. Virology **50:**396–403.
12. **Sonenshein, A. L., B. Cami, J. Brevet, and R. Cote.** 1974. Isolation and characterization of rifampin-resistant and streptolydigin-resistant mutants of *Bacillus subtilis* with altered sporulation properties. J. Bacteriol. **120:**253–265.
13. **Sumida-Yasmumoto, D., and R. H. Doi.** 1977 *Bacillus subtilis* ribonucleic acid polymerase mutants conditionally temperature sensitive at various stages of sporulation. J. Bacteriol. **129:**433–444.
14. **Taylor, T., and H. Weissbach.** 1965. Radioactive assay for serine transhydroxymethylase. Anal. Biochem **13:** 80–84.
15. **Vogel, H. J., and E. E. Jones.** 1970. Acetylornithine δ-amino transferase (*Escherichia coli*). Methods Enzymol. **17:**260–264.
16. **Wainscott, V. J., and J. F. Kane.** 1976. Dihydrofolate reductase in *Bacillus subtilis*, p. 208–213. *In* D. Schlessinger (ed.), Microbiology—1976. American Society for Microbiology, Washington, D.C.
17. **Wozny, M. E., H. N. Carnevale, and E. E. Jones.** 1975. Alteration of regulation of arginine biosynthesis in *Escherichia coli* W by mutation to rifampin resistance. Biochim. Biophys. Acta **383:**106–116.