Nitrosoguanidine Sequential Mutagenesis Mapping of *Mycobacterium tuberculosis* Genes

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Nitrosoguanidine-induced mutations occur at higher frequencies at the replication region than at other nonreplicating regions of the chromosome. Cultures of *Mycobacterium tuberculosis* synchronized with phenylethanol were used to determine the order of replication for 10 genes controlling drug resistance. Use of *M. tuberculosis* provided a 10-h replication map with good resolution because of the slow rate of deoxyribonucleic acid replication. The direction of chromosome replication could not be determined, but this study indicated no pause between rounds of deoxyribonucleic acid replication in a rich medium.

Genetic studies with *Mycobacterium tuberculosis* are difficult, due to the slow growth rate and pathogenicity of this species. Resistance of tubercle bacilli to antitubercular agents is a particular problem because of the long periods of chemotherapy. Applying genetic techniques to the study of this pathogen may help in overcoming this problem. Since no previous genetic map exists for the chromosome of this important pathogen, our aim is the application of sequential mutagenesis (2) to obtain the order of replication of genes for resistance to some antitubercular agents used for chemotherapy. Genes for nutritional requirements were not used because auxotrophic strains of *M. tuberculosis* have not been isolated due to lack of a chemically defined medium that would support good growth.

MATERIALS AND METHODS

Bacterial strain. *M. tuberculosis* H37Rv was used in all experiments. A high-containment laboratory was used at biosafety level 3, as recommended by the Office of Biosafety, Centers for Disease Control, Atlanta, Ga.

Medium. Middlebrook and Cohn 7H-10 agar (BBL Microbiology Systems, Baltimore, Md.) containing 0.5% glycerin and oleic albumin dextrose catalase enrichment (GIBCO Laboratories, Grand Island, N.Y.) was used for growth determinations and drug susceptibility studies. For studies requiring a liquid medium, Middlebrook and Cohn 7H-9 (Difco Laboratories, Detroit, Mich.) containing 0.05% Tween 80 and albumin dextrose catalase enrichment (Difco Laboratories) was used.

Growth conditions. All cultures were incubated at 37°C. When shaking of cultures was required, a Precision Scientific model 50 shaking water bath was used at approximately 100 oscillations/min. Growth

measured by turbidity was obtained at 650 nm by using a Coleman Junior II model 6/20 spectrophotometer.

Chemical and inhibiting agents. *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) and 2-phenylethanol (PEA) were obtained from Sigma Chemical Co., St. Louis, Mo. Isoniazid, cycloserine, and capreomycin sulfate were obtained from Lilly Laboratories, Indianapolis, Ind. The dihydrostreptomycin used was from Merck & Co., Inc., Rahway, N.J.; the kanamycin and amikacin were from Bristol Laboratories, Syracuse, N.Y.; the ethionamide was from Ives Laboratories, New York, N.Y.; the rifampin was from Ciba-Geigy Corp., Summit, N.J.; and the pyrazinamide was from Aldrich Chemical Co., Milwaukee, Wis. Ethambutol was obtained from Lederle Laboratories, Pearl River, N.Y.

Minimal inhibitory concentrations. Minimal inhibitory concentrations of drugs for *M. tuberculosis* were determined on 7H-10 agar and are described in Table 1 for all drugs tested. The minimal inhibitory concentration is defined as the lowest concentration of each drug which prevented growth of the bacteria. For mutagenic studies, minimal inhibitory concentration determinations were made with the highest concentration of organisms possible in the growth medium ($>10^8$ cells/ml). Preparations of antimicrobial agents and media for studies with mycobacterial species are described by David (3) and Vestal (9).

Optimal conditions for NTG treatment. NTG mutagenesis to drug resistance in *M. tuberculosis* was determined by using different conditions for all drugs. These determinations were done with nonsynchronized cultures to ascertain maximum conditions for sequential mutagenesis and isolation of resistant strains. It had been determined in preliminary experiments that NTG mutagenesis was optimal in 0.2 M Tris-maleate buffer (TMB) at pH 6.0. The NTG solutions were freshly prepared before each use. The effect of NTG at 50, 100, 200, and 500 µg/ml in TMB at pH 6.0 was tested with 30- and 90-min exposure

TABLE 1. NTG-induced and spontaneous mutation frequencies to drug resistance in *M. tuberculosis* strain H37Rv

Drug	Minimal inhibitory concn ($\mu\text{g/ml}$)	Mutation frequency	
		Spontaneous	NTG
Amikacin	0.5	5.0×10^{-6}	4.5×10^{-5}
Capreomycin	5.0	1.5×10^{-6}	1.5×10^{-5}
Cycloserine	30.0	7.0×10^{-8}	8.5×10^{-7}
Ethambutol	7.0	7.0×10^{-8}	1.4×10^{-7}
Ethionamide	5.0	7.0×10^{-8}	1.1×10^{-5}
Isoniazid	0.2	3.8×10^{-6}	2.9×10^{-5}
Kanamycin	3.0	5.9×10^{-7}	2.4×10^{-6}
Pyrazinamide	100.0	9.9×10^{-7}	3.2×10^{-6}
Rifampin	0.5	2.6×10^{-7}	1.5×10^{-5}
Streptomycin	5.0	3.1×10^{-6}	2.6×10^{-5}

times. After exposure, the cells were centrifuged at $2,000 \times g$ for 30 min, suspended in TMB, and centrifuged again. The cells were then suspended in 7H-9 broth and diluted. A 0.1-ml quantity of each dilution in the series was inoculated to 7H-10 agar plates to determine the lethal effect of these concentrations of NTG. The undiluted broths were then incubated for 7 days without shaking for phenotypic expression of NTG-induced mutations. After incubation, the broths were inoculated undiluted to a series of 7H-10 agar plates containing rifampin, streptomycin, and isoniazid, which were incubated for 21 days. The frequencies of mutants were determined by the formula: mutant frequency = M/N , where M is the number of mutants, and N is the total number of bacteria. Mutants per 10^6 survivors = mutant frequency $\times 10^6$.

Induced and spontaneous drug resistance frequencies. Frequencies of spontaneous and NTG-induced drug resistance were determined by growth in 7H-9 broth to a cell concentration of at least 10^6 cells per ml. The culture was divided into two equal portions, centrifuged, and washed with TMB; 100 μg of NTG per ml in TMB (pH 6) was added to one part, and TMB alone was added to the second part. After 30 min, both sets were centrifuged twice, and the cells were washed with TMB. Cells were suspended in distilled water containing 0.05% Tween 80 to 1/10 the original volume. One-tenth-milliliter volumes of the suspensions were inoculated to 7H-10 agar containing a drug concentration established by minimal inhibitory concentration determinations. Serial 10-fold dilutions of the cells were made, and 0.1 ml of a 10^{-6} dilution was inoculated onto 7H-10 agar without drugs for counts of viable units.

PEA synchronization. Cultures of *M. tuberculosis* were synchronized by a modification of the method described by Altenbern for *Staphylococcus aureus* (1). A 0.4% PEA concentration caused reversible inhibition of growth of *M. tuberculosis* strain H37Rv when it was grown to an optimal density of 0.2 in 7H-9 broth. A 300-ml volume of a PEA-containing culture (approximately 2×10^7 colony-forming units per ml) was incubated without shaking for 24 h at 37°C , then centrifuged, washed with buffer, and centrifuged again, and 300 ml of fresh prewarmed 7H-9 broth was added to the sediment. Duplicate samples were withdrawn,

and the 10^{-4} , 10^{-5} , and 10^{-6} dilutions were plated. This sample was designated time zero, and samples were taken every 60 min thereafter for a period of 24 h. A nonsynchronized control culture was treated in the same manner for growth rate determinations. Both cultures were incubated with shaking during the sampling period.

Sequential mutagenesis. The PEA-synchronized cells were incubated with shaking at 37°C upon reinitiation of DNA synthesis. At 60-min intervals, 10-ml samples were withdrawn for a period of 10 h. If more than one generation was to be studied, samplings were continued for 24 h. The samples were washed free of the 7H-9 broth by centrifugation. Ten-milliliter volumes of prewarmed TMB (pH 6) containing 100 μg of NTG per ml were added to the sediment and incubated at 37°C for 30 min. Cold TMB was added, and the suspensions were centrifuged, washed, and then recentrifuged. A 10-ml amount of 7H-9 was added to the sediment and incubated at 37°C without shaking for 7 days. The cells were then washed free of the 7H-9 broth, and 10 ml of 0.05% Tween 80 was added; then, 10^{-5} and 10^{-6} dilutions were plated for determination of viable units. Undiluted suspensions were inoculated onto a series of drug-containing 7H-10 agar plates and incubated for 21 days at 37°C for detection of mutants.

RESULTS

Optimal conditions for NTG mutagenesis. It had previously been determined that a 20-min exposure with NTG in 0.2 M TMB (pH 6) was optimal for mutagenesis of some rapidly growing species of mycobacteria (6, 7). Increasing concentrations of NTG caused increasing loss of viability for H37Rv, as represented in Fig. 1. The lethal effect was greater after 90 min of exposure than after 30 min. By using 30% survival as an acceptable level for mutagenic experiments, 100 μg of NTG was optimal at 30 min for *M. tuberculosis*. The mutagenic effect of

FIG. 1. Effect of NTG on *M. tuberculosis* strain H37Rv. Cells were treated with different concentrations of NTG in Tris-maleate buffer (pH 6) for 30 min (●) and 90 min (○).

different concentrations of NTG was also established as shown in Fig. 2. More mutants resistant to drugs were produced with 500 $\mu\text{g}/\text{ml}$ than with lower concentrations; however, because of the lethality of this concentration, 100 $\mu\text{g}/\text{ml}$ was used for all sequential mutagenesis experiments.

Mutant frequencies. The frequency of mutants due to spontaneous mutations to drug resistance is shown in Table 1 and compared with induced mutations. NTG increased the mutant frequencies 10- to 100-fold.

Synchronization. *M. tuberculosis* was inhibited reversibly in 7H-9 broth by 0.4% PEA. This concentration was previously used for synchronization of *M. phlei* (5) and *M. smegmatis* (7). The results of PEA-induced synchronization of *M. tuberculosis* are shown in Fig. 3B. Conditions for synchronization must include incubation without shaking during exposure to PEA for 24 h. A 10.5-h initial plateau followed by a doubling of cell numbers by 16 h and a second plateau indicated good synchrony of growth through at least one generation. The generation time of *M. tuberculosis* strain H37Rv under

FIG. 2. Mutagenesis to resistance of *M. tuberculosis* strain H37Rv to (A) rifampin, (B) streptomycin, and (C) isoniazid with different concentrations of NTG in TMB at pH 6 for 30 min (●) and 90 min (○).

FIG. 3. (A) Growth rate of nonsynchronized *M. tuberculosis* strain H37Rv in 7H-9 broth with albumin dextrose catalase enrichment. Abbreviations: g, mean doubling time = $0.693 (t_z - t_0) / \log_{10} Z - \log_{10} Z_0$ (2.303), where Z is the number of viable cells per milliliter at time t . (B) PEA (0.4%) synchronization of *M. tuberculosis* strain H37Rv in 7H-9 broth with albumin dextrose catalase enrichment. Undiluted culture was incubated without shaking in the presence of PEA for 24 h. The culture was washed twice to remove the PEA.

nonsynchronous conditions is 18 h and 14 min in 7H-9 broth (Fig. 3A).

Sequential mutagenesis. Sequential mutagenesis experiments with a synchronized culture of *M. tuberculosis* strain H37Rv indicated that a period of 10 h was required for DNA replication. Figures 4-6 show the peaks of optimal mutagenesis for resistance to nine antitubercular agents for the first round of DNA replication. The maximum frequencies of resistance to isoniazid, capreomycin sulfate, and pyrazinamide occurred at 4, 7, and 5 h, respectively (Fig. 4). Increased frequencies of resistance to cycloserine, kanamycin, and ethionamide occurred at 9, 4, and 3 h, respectively (Fig. 5). Figure 6 shows that the maximum frequencies of resistance to ethambutol, amikacin, and streptomycin oc-

FIG. 4. Sequential mutagenesis of *M. tuberculosis* strain H37Rv. Resistance to (A) 0.2 μ g of isoniazid per ml, (B) 5 μ g of capreomycin per ml, and (C) 100 μ g of pyrazinamide per ml was induced.

curring at 1, 4, and 8 h, respectively. The detection of rifampin mutants through more than one generation is shown in Fig. 7. There was no pause between successive rounds of DNA replication as indicated by the separation of the peaks by exactly 10 h. Synchronization by PEA as shown by this experiment with rifampin resistance was good for H37Rv beyond the first generation.

The replication map derived from these experiments (Table 2) is a 10-h map on which are found the sites of the 10 drug resistance markers. The genes for the aminoglycosides, amikacin and kanamycin, replicated at 4 h with isoniazid resistance. This cluster of three genes was the only cluster, whereas the other seven genes replicated at seven different times.

DISCUSSION

The human pathogen *M. tuberculosis* has rarely been used for genetic experiments. Therefore, little is known about the genetic makeup of its chromosome. The order of replication presented here cannot be interpreted as the actual sequence of chromosome replication because the data may be interpreted two ways. The chro-

mosome may replicate unidirectionally or bidirectionally, a problem which has been discussed previously in sequential mutagenesis experiments with *Escherichia coli*. Cerda-Olmedo et al. (2) interpreted their results as unidirectional replication starting from a fixed origin because they were able to compare the replication map to a genetic map produced by conjugation. Hohl-feld and Vielmetter (4), using two different methods of synchronization, interpreted their results as replication beginning at a fixed origin and proceeding bidirectionally to the terminal. They determined that certain markers that replicated at the same time period by sequential mutagenesis were actually found opposite on the genetic map produced by conjugation. No genetic map exists for mycobacteria obtained by conjugation experiments. Therefore, analyses such as those permitted with *E. coli* were not possible with mycobacteria used for sequential mutagenesis experiments (5, 8).

The absence of a pause between rounds of DNA replication in these experiments agrees with published results of sequential mutagenesis where second-generation mutagenesis was studied (2, 11). The synchronization curve produced

FIG. 5. Sequential mutagenesis of *M. tuberculosis* strain H37Rv. Resistance to (A) 30 μ g of cycloserine per ml, (B) 3 μ g of kanamycin per ml, and (C) 5 μ g of ethionamide per ml was induced.

FIG. 6. Sequential mutagenesis of *M. tuberculosis* strain H37Rv. Resistance to (A) 7 μ g of ethambutol per ml, (B) 0.5 μ g of amikacin per ml, and (C) 5 μ g of streptomycin per ml was induced.

FIG. 7. Sequential mutagenesis of *M. tuberculosis* strain H37Rv with change of resistance to 0.5 μ g of rifampin per ml during two generations.

(Fig. 3) demonstrated that DNA replication takes 10.5 h to complete one round. This extremely slow rate of DNA replication correlates with the slow division time of 18 h. The results agree with the previous synchronization of H37Rv by Wayne (10), who utilized selection of

TABLE 2. Sequential mutagenesis of *M. tuberculosis* strain H37Rv

Drug	Replication time (h)
Amikacin	4
Capreomycin	7
Cycloserine	9
Ethambutol	1
Isoniazid	4
Kanamycin	4
Pyrazinamide	5
Rifampin	2
Streptomycin	8
Ethionamide	3

uniform cells by sedimentation in terms of DNA synthesis and growth rate. The slow replication rate makes *M. tuberculosis* well suited for studies of sequential mutagenesis. The distribution of the 10 markers over the entire length of the chromosome made resolution unnecessary, although it will be possible when more markers are tested to increase resolution by taking samples at intervals less than 1 h. Experiments with this procedure may yield information on multi-step drug resistance seen in *M. tuberculosis* strains. This may be accomplished by using drug-resistant strains of *M. tuberculosis* for sequential mutagenesis and determining whether higher levels of resistance involve genes that replicate at time periods different from those of lower levels.

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