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Effect of Temperature on the Rate of the Transparent to Opaque Colony Type Transition in *Mycobacterium avium*

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The results of drug susceptibility tests were found to be affected by changes that occur spontaneously in populations of *Mycobacterium avium* maintained in the laboratory. Because the transparent colony type variant was resistant to antituberculosis chemotherapeutic agents and the opaque colony type variant was usually susceptible to these agents, the transition of transparent to opaque colony type was investigated. The rate of the transition was found to be temperature dependent and, in agreement with a previous report, was found to be about 10^{-4} to 10^{-5} per generation at 37 C. Reversion was found to occur at a rate of 10^{-6} to 10^{-7} at 37 C. The mutation rate from susceptibility to resistance to rifampin, kanamycin, and erythromycin was about 10^{-8} to 10^{-9} mutations per bacterium per generation. Judged from our data, the high rate of the transparent to opaque variation was not caused either by mutator effects or by the occurrence of extrachromosomal genes in these bacteria, but could have been due to selective mechanisms still incompletely understood.

Recent investigations on colony type variations in *Mycobacterium avium* showed that the transition of transparent colonies to opaque colonies occurred at the high frequency of about 10^{-4} to 10^{-5} mutations per bacterium per generation; reversions were stated to occur infrequently, but the frequency of the opaque to transparent transition was not established (13). Earlier investigations showed that the transparent variants differed from the opaque variants in several properties. The transparent variant tolerates higher temperatures of growth (16, 17), is virulent to experimental animals (15, 19, 25) and is resistant to most antituberculosis chemotherapeutic agents (24). Because it is possible that the transparent variant is the causative agent of disease in man, it is of importance to understand the mechanism of the transparent to opaque transition.

The finding that the spontaneous mutation frequency of the transparent to opaque variation was unusually high led McCarthy (13) to propose that the transition could be caused either by the loss or gain of satellite deoxyribonucleic acid (plasmids, episomes, temperate bacteriophages), even though the reverse transition was seen on occasion. It is the purpose of this report to show that the high frequency of the transparent to opaque transition is not a measure of the spontaneous mutation frequency in these bacteria because the temperature of growth exerts a selective pressure upon the two variants.

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MATERIALS AND METHODS

Bacteria. The strain T-931-72 of *M. avium*, serotype 2, which was used in these investigations, is the same strain used in previous investigations on the effect of ultraviolet (UV) light radiation in the mycobacteria (6). The strain is maintained in the mycobacterial culture collection of the Center for Disease Control, Atlanta, Ga.

Media. The bacteria were grown in liquid Middlebrook and Cohn 7H9 medium (Difco) containing 0.05% of Tween 80 and the ADC enrichment. Surface plate counts were performed on Dubos oleic acid agar (DMA) containing the OAA enrichment (Difco). Middlebrook and Cohn 7H-10 agar (Difco) containing OADC enrichment was used in drug susceptibility determinations. MacConkey agar (Difco) was prepared from the powdered base without modification. For dye susceptibility tests, 0.01% of the various dyes was incorporated into DMA.

Cloning. The transparent variant yields opaque variants at high frequency (13). When plates of the pure transparent variant are left at room temperature or in the incubator for several weeks, the

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opaque variant appears as papillae on the transparent colonies that are eventually overgrown. Pure transparent clones were therefore obtained by serial transfers of single colonies from plates incubated for 2 to 3 weeks at 37 C. Although the opaque variant was reported stable (7), it was cloned in the same manner. Suspensions of both variants after cloning were prepared by washing the surface of the plates with sterile distilled water. The water suspensions were then stored at −20 C. When inocula were needed, the frozen suspensions were thawed and then were streaked onto DMA plates. After incubation at 37 C for 21 days, isolated colonies were suspended in distilled water to an optical density of about 0.1 at 650 nm by using a Coleman Jr. model 6/20 spectrophotometer.

Susceptibility to growth inhibitory agents. The drug susceptibility tests were modified versions of the proportion method of Canetti et al. (4, 23).

Temperature relationships. Temperature-growth response measurements were performed in a temperature gradient block (Lab-Line Instruments, Inc., Melrose Park, Ill.) set for a temperature range of 22 to 42 C. The temperature gradient block had 19 duplicate wells, which made it possible to measure growth at 19 different temperatures. The temperature differential between two contiguous wells was approximately 1 C in this instrument. The built-in vibrating mechanism was set at 60 strokes/min.

Growth measurements. The specific growth rate constants ($days^{-1}$) at each temperature were calculated from the formula $k = (\ln N_t - \ln N_0)/(t - t_0)$, where N_t and N_0 are the number of cells at times t and t_0, respectively. Growth was estimated either turbidimetrically at 650 nm or by surface plate counts on DMA plates. Plots of the cell density against the number of viable cells were obtained and were used when appropriate.

The temperature characteristics (μ) were obtained from the linear portion of Arrhenius plots by using the formula $\mu = 2.303\ R\ T_1\ T_2\ (\log k_2 - \log k_1)/(T_2 - T_1)$, where k_1 and k_2 are the growth rate constants at the absolute temperatures T_1 and T_2, respectively.

Mutation frequencies. The mutation frequencies were estimated from fluctuation tests (12) by using the equation $r = \alpha N_t \ln (N_t\ C\ \alpha)$, where r is the average number of mutants, N_t is the total number of bacteria, C is the number of replicate cultures, and α is the mutation rate. Mutation rates ($\alpha = \mu/n$) from data on the frequency of mutants in respect to the age of the culture were obtained using the formula $\mu = 2 \ln (M_t/N_t - M_0/N_0)/n$, where M_t/N_t and M_0/N_0 are the frequency of mutants at times t and t_0, respectively (21). The number of generations, n, was obtained from $n = (\log N_t - \log N_0)/0.301$. Both procedures had been applied before to the mycobacteria (5, 13).

RESULTS

Susceptibility to inhibitory agents. In agreement with a previous report (24), the transparent variant of *M. avium* was found to be resistant to all antitubercular chemotherapeutic agents and several dyes, whereas the opaque variant was found to be susceptible to most of these inhibitory agents (Table 1). These observations, coupled with a recent report indicating that the transparent to opaque transition occurred at very high frequencies (13), indicated that the study of the factors that may contribute to population changes in *M. avium* was necessary to properly interpret the results of drug susceptibility testing of these bacteria.

Temperature relationships. Figure 1 is an Arrhenius plot of the transparent and opaque variants. The data showed that the specific growth rate of the two variants increased at approximately the same rate from 22 to 30 C. However, the opaque variant reached its maximal growth rate at 30 to 32 C, whereas the transparent variant reached its maximal growth rate at 35 to 37 C. Further, the maximal growth temperature of the opaque variant was 39 to 40 C and the maximal growth temperature of the transparent variant was above 42 C. The specific growth rate constant of the transparent variant was consistently higher throughout the temperature ranges studied (Fig. 1).

The above data were obtained from cultures inoculated with clones of the two variants. As the cultures grew at the indicated temperatures, the bacterial populations changed. For example, after 8 days of incubation the frequencies of opaque variants in cultures initiated with pure clones of transparent variants were 0.8, 0.6, and 0.1% at 25, 36, and 41 C, respectively; in another experiment the frequencies were 0.78, 0.65, and 0.43% at 34, 36, and 39 C, respectively. Figure 2 illustrates the emergence of opaque variants in batch cultures started with pure clones of the transparent variant. The ratios of opaque to transparent variants

TABLE 1. *Effects of inhibitory agents on colony type variants of M. avium strain T-931-72*

Inhibitory agent	Concn (μg/ml)	Resistant bacteria (%)	
		Opaque	Transparent
Isonizid	1	100	100
Streptomycin	5	0	100
Erythromycin	5	0	100
Rifampin	5	0	100
Kanamycin	5	0	100
Malachite green	100	11	100
Methyl violet	100	7	72
Pyronin B	100	50	100
Biebric scarlet	100	100	41
Eosin Y	100	95	100
MacConkey		0	44

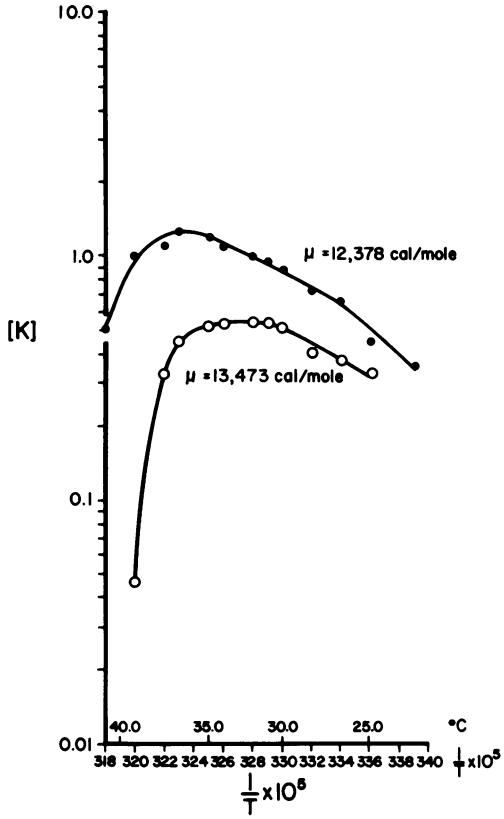

FIG. 1. *Growth of the transparent variant* (●) *and of the opaque variant* (○) *as a function of the temperature of incubation.*

varied from experiment to experiment; however, in all experiments a relationship between these ratios and the temperature of incubation was consistently noted. It is possible that in a continuous culture condition, the shifts in the frequency of the variants may be studies more accurately.

The opaque variants were found in populations initiated with clones of the transparent variant at any temperature of incubation; on the other hand, in cultures initiated with clones of the opaque variant, the transparent variant was observed only rarely.

Transparent to opaque variation. From the above data, it seems clear that the relative abundance of each variant in a given population depends upon the temperature of growth and the age of the culture. This notion was substantiated in experiments in which the frequency of transparent to opaque variation was calculated at distinct temperatures. As shown in Fig. 3, the rate of variation (α) was a reciprocal function of the temperature. The rate of the variation at 37 C was estimated graphically to be about 4.6×10^{-4}, which is in agreement with a previous report (13). Clearly, if the value of α is temperature dependent, then it can not be assumed to represent the rate of spontaneous mutations in the locus, or the loci, that control the transparent phenotype.

Opaque to transparent variation. The opaque variant has been considered stable (7), and in studies on the genetic mutation in *M. avium*, McCarthy (13) briefly mentions that the opaque to transparent variation was observed seldom. Whether this transition does indeed occur is important in determining the validity of the hypothesis that the transition is under the control of extrachromosomal genes.

Although a few opaque colonies are easily seen on a lawn of transparent colonies, it is not possible to observe a few transparent colonies on a lawn of opaque colonies. Furthermore, if a clone of opaque variants yields a few transparent variants, on surface plate counts, the latter will be washed out by dilution. Therefore, it is possible that the opaque to transparent variation is reported to be seen only on occasion because of the difficulty in detecting the transparent colonies in cultures initiated with a clone of opaque variants. In an attempt to resolve this question, we performed fluctuation analyses taking advantage of the fact that the transparent variants are uniformly resistant to most chemotherapeutic agents (24). The rationale of the experiments was that the transparent variants derived from a clone of opaque variants would grow on drug-containing media, whereas the majority of the population made of opaque variants would be inhibited. Thus, by using drugs as nonessential selective agents, the chances of demonstrating the opaque to transparent variation could be enhanced. When small, identical cultures were prepared for a fluctuation test and were then plated on drug media, we observed large numbers of transparent colonies on the plates. Because some of the identical cultures yielded almost confluent growth of the transparent variety, no counts could be obtained. The experiments were then repeated by plating dilutions of the identical cultures, and the results of these tests are depicted in Table 2. The experiments demonstrated that, at the temperature used (37 C), opaque variant yielded the transparent variant at a frequency of about 10^{-6} per bacterium per generation.

Table 2 shows the results of experiments in which no opaque, drug-resistant colonies were seen. However, when the cultures were plated undiluted, a few opaque colonies were seen on

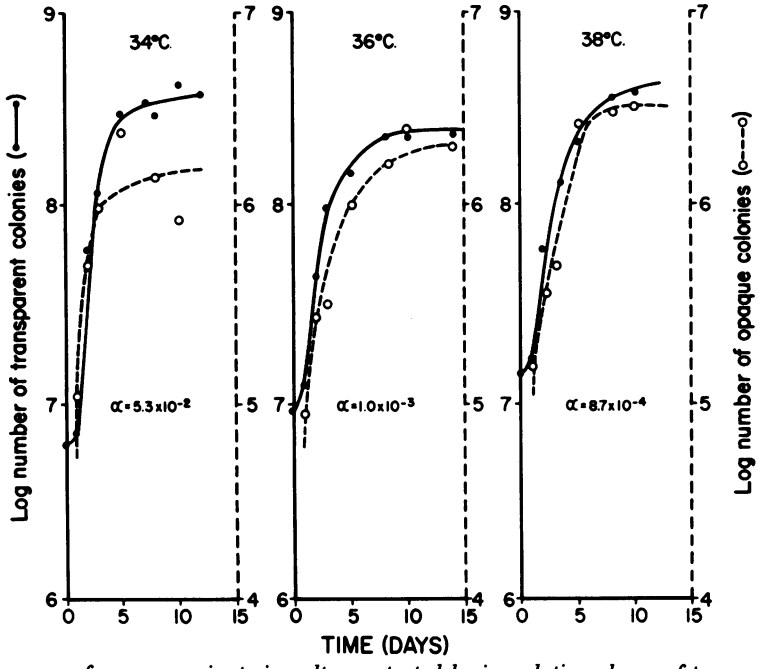

FIG. 2. *Emergence of opaque variants in cultures started by inoculating clones of transparent variants. Note that the scales differ by 2 log units.*

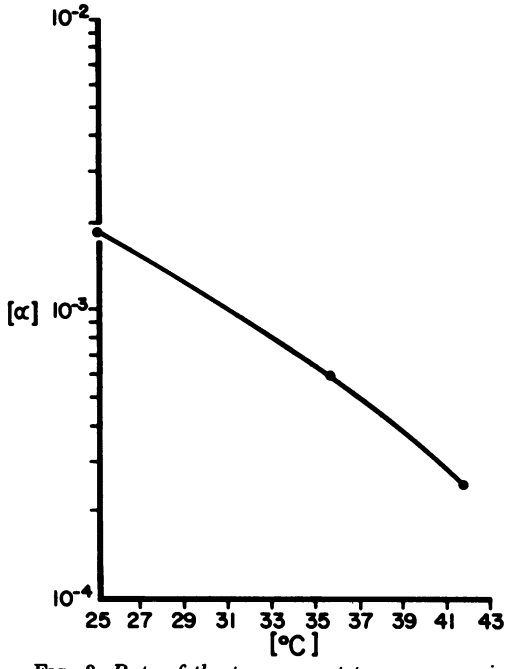

FIG. 3. *Rate of the transparent to opaque variation (α) as a function of the temperature of incubation.*

the media containing rifampin, kanamycin, and erythromycin. The average number of drug-resistant mutants was 0.15, 0.1, and 0.1, and the susceptibility to resistance mutation frequencies was, respectively, 6.5×10^{-9}, 6.0×10^{-9}, and 6.0×10^{-9} mutations per bacterium per generation.

U-tube experiments. Braun and his associates (3) demonstrated that the frequency of smooth, rough, and mucoid variants in cultures of *Brucella abortus* was the result of the interplay of spontaneous mutations and of selection directed by the accumulation in the medium of metabolites that favored one variant over another. Thus, for example, the smooth variants excreted D-alanine towards which they were partially susceptible, and the rough variants that were more resistant to D-alanine than the parental organism were then favored and tended to accumulate in the population. To verify whether a similar condition did occur in cultures of *M. avium,* we performed U-tube experiments (Table 3). Our data did not demonstrate that either variant produced metabolites that would increase or decrease the growth rate of the other, or would otherwise influence the relative abundance of either variant.

DISCUSSION

Atwood et al. (1) defined selection as any factor other than mutation that influences the frequency of genetic variants in bacterial populations. On the other hand, as pointed out by Luria and Delbruck (12), the measurement of

TABLE 2. *Fluctuation analysis of the opaque to transparent transition*

Determinants	Nonessential selective agents (μg/ml)			
	Rifampin	Streptomycin	Kanamycin	Erythromycin
No. of cultures (C)	20	20	20	20
Vol of cultures (ml)	5.0	5.0	5.0	5.0
Vol of sample[a]	0.1, 10^{-1}	0.1, 10^{-1}	0.1, 10^{-1}	0.1, 10^{-1}
No. of transparent colonies	Frequency of plates with the indicated no. of variants			
9	1	0	0	0
10	1	0	0	0
11	1	0	0	0
12	1	0	0	0
14	1	0	1	1
17	0	0	2	0
18	2	0	2	1
19	0	0	2	1
20	1	1	0	0
21	1	0	1	0
22–26	3	2	4	1
27–30	1	3	0	2
31–35	1	3	1	4
36–40	0	3	2	2
41–45	0	0	1	3
46–50	3	0	2	1
51–55	2	3	0	1
56–60	1	1	0	0
61–65	0	2	0	0
66–70	0	0	0	0
71–75	0	0	0	0
76–80	0	1	1	0
81–85	0	0	0	1
86–120	0	1	1	0
121–140	0	0	0	2
Avg no. of variants/0.1 ml (r)[b]	287.5	552.0	344.0	456.0
Bacteria/0.1 ml (N_t)[b]	2.27×10^7	2.27×10^7	2.27×10^7	2.27×10^7
Mutation rate (α)	1.9×10^{-6}	2.8×10^{-6}	2.2×10^{-6}	2.8×10^{-6}

[a] The inoculation of the undiluted growth from the 20 identical cultures yielded confluent growth in most plates; the data shown was obtained by inoculating the plates with 0.1 ml of a 10^{-1} dilution of each of the identical cultures. The calculations were made after the appropriate corrections were performed. The 20 identical cultures were started by inoculating an average of about 100 cells.

[b] $r = \alpha \cdot N_t \cdot \ln (N_t \cdot C \cdot \alpha)$.

TABLE 3. *Frequency of opaque variants in the left and right arms of U-tubes which were inoculated with clones of transparent (Tr.) or opaque (Op.) variants, or were uninoculated (Uninoc.)*

U tube		Frequency of opaque variants/ml			
		Day 0		Day 10	
Left	Right	Left	Right	Left	Right
Transparent	Uninoculated	3.9×10^{-3}	0	3.5×10^{-4}	0
Uninoculated	Opaque	0	1.0	0	1.0
Transparent	Opaque	1.3×10^{-3}	1.0	2.0×10^{-4}	1.0

mutation rates by the fluctuation analysis of bacterial populations postulates that none of the genetic variants has a selective advantage. Our investigations showed that the high frequency of the transition of the transparent colony to the opaque colony in *M. avium* was dependent upon the temperature of growth and that the rate of the transition was a reciprocal

function of the temperature of growth. Therefore, the previously reported rate of 10^{-4} to 10^{-5} for spontaneous mutations per bacterium per generation (13) is higher than the true mutation frequency. Although the temperature of growth was unquestionably a selective force, there may have been other factors which also influenced the transition. For example, the transition could be analogous to the environmentally induced transition from the flagellated to the nonflagellated state in *Salmonella typhimurium* (18, 21).

In addition to selection, we have also considered two other hypotheses: (i) that the high frequency of transparent to opaque variation, and associated phenotypic changes (such as susceptibility to drugs and pathogenicity) was caused by mutator effects (2, 8, 9, 10, 22); and (ii) that the transparent variant could harbor extrachromosomal genes. We consider these hypotheses unlikely for the following reasons.

Mutator effects are usually generalized, and certainly the changes in drug susceptibility patterns associated with the colonial type variation could be caused by mutations occurring at high frequencies in several genes. The finding that the mutation rates from susceptibility to resistance measured in the opaque variant were about 10^{-8} to 10^{-9} for three distinct markers (rifampin, kanamycin, erythromycin) seems to rule out mutator effects. Furthermore, mutator strains that are susceptible to UV radiation in consequence of deficient deoxyribonucleic acid repair have been described (2, 10, 11, 20). As judged from previous reports, the susceptibility of *M. avium* to UV radiation is within the range expected from the structure of its deoxyribonucleic acid (6), and these bacteria were shown to be capable of repairing the UV-induced lesions (6, 14).

The possibility that the phenotypic change could be caused by loss of extrachromosomal genes was also investigated. Since the loss of a plasmid is permanent, if the transparent phenotype was controlled by a plasmid whose loss would cause the phenotype to change to opaque, the opaque to transparent variation ought not to occur. However, as shown in our report, the reversion did occur at the relatively high frequency of 10^{-5} to 10^{-6} at 37 C, which argues against the hypothesis.

There is seldom agreement between drug susceptibility tests in vitro and the results of chemotherapy of patients suffering from disease caused by *M. avium* and *M. intracellulare*. It is therefore of practical interest to elucidate the mechanism of the transparent to opaque variation, which has been associated with variations in pathogenicity (7, 15) and the drug susceptibility patterns (24). As shown here, the transparent variant was resistant to chemotherapeutic agents and a variety of other toxic agents. It is therefore possible that the cell surface of the transparent variant excludes toxic agents more efficiently than the cell surface of the opaque variants. Whether this exclusion may relate to the pathogenicity of the transparent variant is a matter of speculation.

Our studies seem to show why drug susceptibility tests as performed currently may indicate susceptibility to chemotherapeutic agents in vitro in a disease condition that is mostly refractory to chemotherapy; and our results indicate the need for further investigating the transparent-opaque variation by using, perhaps, continuous culture systems.

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