Artificial Induction of an Organism Similar to *Mycobacterium triviale* from *Mycobacterium gordonae* by Successive Ultraviolet Irradiations

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An organism phenetically indistinguishable from *Mycobacterium triviale* was isolated from *Mycobacterium gordonae* (ATCC 14470) by successive ultraviolet irradiations after three selection steps. From the parent (a smooth-colony-forming, scotochromogenic strain) were isolated: (i) photochromogenic mutants; (ii) rough-colony-forming mutants; and (iii) non-photochromogenic (pigmentless) mutants. In the third step of mutation, some mutants showed simultaneous changes of other characters, e.g., loss of the ability to utilize several carbon sources. After ultraviolet irradiation, mutant colonies comprised 1 to 2 per 10^3 surviving colonies. It was impossible to isolate non-photochromogenic mutants directly from parent scotochromogenic bacteria. Mutants could be isolated only from photochromogenic mutants. This is in marked contrast to a previous work in which non-photochromogenic mutants were isolated directly from parent, scotochromogenic *Mycobacterium scrofulaceum*.

An important purpose of taxonomy is to show phylogenetic relationships among microorganisms. If possible pathways of evolution could be reproduced experimentally, investigations would be facilitated.

As an aid to the taxonomic study of mycobacteria, attempts to artificially produce one species from another species were begun with *Mycobacterium gordonae*, a slowly growing, scotochromogenic species that occurs very commonly in the environment (10) and has been known previously as a tapwater scotochromogen (8, 14). An organism was experimentally produced that is similar to *M. triviale* and to another organism resembling *M. terrae*.

MATERIALS AND METHODS

Strains. The parent, *Mycobacterium gordonae* Bojalil et al. (1), strain T-12109, was received from the American Type Culture Collection, Rockville, Md., as ATCC 14470. Strains of *M. triviale* Kubica et al. (2), *M. terrae* Wayne (13), and *M. nonchromogenicum* Tsukamura (7) were used for comparison. The sources of these strains were given previously (9). The test strains were cultivated on Ogawa egg medium slants at 37°C for 14 days.

Tests. The tests used were described previously (9).

Ultraviolet irradiation. Ultraviolet irradiation was used for inducing mutations in *M. gordonae* T-12109. Growing organisms taken from Ogawa egg medium slants were homogenized by 5 min of shaking in a flask containing glass beads; a 10- or 1-mg (moist weight)/ml suspension was then prepared (organisms suspended in 0.067 M phosphate buffer [pH 7.1] containing 0.1% [vol/vol] Tween 80). A series of 10-fold dilutions was prepared (down to 10^{-7}) from the sus-

pension. As an aid for counting the number of viable bacteria before irradiation, a 0.02 ml sample of each 10^{-4} to 10^{-7} dilution was inoculated into five tubes of Ogawa egg medium by using a spiral loop. The original suspension (10 ml) was then placed in a petri dish (9-cm diameter) and irradiated by using a germicidal lamp (National GL-15, Matsushita Electric Company, Osaka; 15 W) at a distance of 1 m for 15 min. During irradiation, the suspension was stirred by rotating the dish by hand for 15 s at intervals of 1 min. The irradiation was carried out in a dark room, to avoid photoreactivation, at room temperature (20 to 25°C). After irradiation, the suspension and a 10^{-1} dilution of it were inoculated into 30 to 50 tubes of Ogawa egg medium with a spiral loop that delivered a 0.02-ml sample. The inoculated tubes were stoppered with rubber caps with pin holes and incubated at 37°C for 14 to 21 days in the dark. Under the conditions used, the number of viable bacteria fell to 10^{-6} to 10^{-7} after irradiation. To test for photochromogenicity or non-photochromogenicity of nonpigmented colonies in the dark, each single colony was inoculated onto two Ogawa egg medium slants, one of which was incubated at 37°C in the dark; the other was exposed to light. After incubation for 14 days, pigmentation of colonies was observed.

Numerical classification. Numerical classification was carried out using the same 88 characters and the same techniques as used previously (9). From the calculated matching coefficients, clustering was determined by a single-linkage method.

Designation of mutants. To simplify the description, photochromogenic mutants, rough-colony-forming (R-type) mutants, and non-photochromogenic (pigmentless) mutants were designated P, R, and N, respectively; the numbers indicate the colony number (mutant number). For example, P1, P2, and P3 represent three photochromogenic mutants isolated from the parent, scotochromogenic strain.

RESULTS

The selection steps after ultraviolet irradiation are shown in Fig. 1. An organism phenetically indistinguishable from *M. triviale* was isolated by steps a, b, and c_1 , and an organism resembling *M. terrae* was isolated by steps a, b, and c_2 .

At step a, photochromogenic mutants were isolated from parent, smooth-colony-forming, scotochromogenic bacteria at a rate of $2.4/10^3$ surviving colonies (27 photochromogenic colonies appeared among 11,160 scotochromogenic surviving colonies). The mutants appeared as pigmentless colonies in the dark, and all were shown to be photochromogenic after incubation under light. Three strains (P1, P2, and P3) of the photochromogenic mutants were tested for their biological and biochemical characters, and one of them (P2) was used for the next ultraviolet irradiation experiment. Rough-colony-forming (R-type) mutants were isolated from the P2 strain at a rate of $1.5/10^3$ surviving colonies (23 per ca. 15,000 surviving colonies). Three strains (P2-R1, P2-R2, and P2-R3) of the R-type photochromogenic mutants were tested for their characters, and two strains (P2-R1 and P2-R3) were used for the next ultraviolet irradiation experiment. Non-photochromogenic mutants were isolated from these strains at rates of 2.4 and $2.0/10^3$ surviving colonies, respectively (8 per ca. 3,300 and 6 per ca. 3,000 surviving colonies, respectively). Fourteen strains of these mutants (P2-R1-N1 to P2-R1-N8 and P2-R3-N1 to P2-R3-N6) were tested for their characters.

In contrast to the parent strain of *M. gordonae*, which is able to utilize glucose, acetate, pyruvate, *n*-propanol, *n*-butanol, and iso-butanol as the sole source of carbon in the presence

of NH_4 -nitrogen, 12 of the 14 mutants lacked the capacity to utilize some of these carbon compounds. One strain (P2-R1-N4) did not utilize any of these carbon compounds; the characteristics of the strain were very similar to those of the *M. triviale* strains (Table 1). The colony of another (P2-R1-N7) exhibited an appearance intermediate between the R-type and the S-type. It resembled the S-type but formed smooth, somewhat dry colonies, although other mutant colonies were rough and dry. The colonies of the parent strain and P mutants were smooth and very wet. The mutant P2-R1-N7 grew more slowly than the other mutants. The other strains showed abundant growth on Ogawa egg medium slants after 7 days, whereas the mutant showed such growth only after 10 days, and its colony appearance was similar to that of *M. terrae*.

A comparison of the characters of the mutants and the parent strain of *M. gordonae* and the reference strains of *M. nonchromogenicum*, *M. terrae*, and *M. triviale* is shown in Table 1.

Other experiments to obtain non-photochromogenic (pigmentless) mutants were also carried out. In one, R-type mutants were isolated and then photochromogenic mutants were isolated from them. Non-photochromogenic mutants were, in turn, isolated from the latter (Fig. 1). The mutants were isolated at every step at a rate of 1 to $2/10^3$ surviving colonies. The R-type, non-photochromogenic mutants thus obtained utilized glucose, acetate, succinate, and pyruvate in the presence of glutamate-nitrogen and utilized *n*-butanol or iso-butanol in the presence of NH_4 -nitrogen.

It was difficult to isolate non-photochromogenic (pigmentless) mutants from S-type photochromogenic mutants, although it could be

FIG. 1. Artificial induction of mutants similar to or resembling *M. triviale* and *M. terrae* from *M. gordonae* by ultraviolet irradiations.

TABLE 1. *Continued*

Character	T-12109	P2	P2-R1	P2-R1-N4	P2-R1/N7	37013	37004	38013	38008	09003
Niacin production	—	—	—	—	—	—	—	—	—	—
Tween hydrolysis at 14 days	+	+	+	+	+	+	+	+	+	+
Catalase (foam height, >45 mm)	+	+	+	+	+	+	+	+	+	+
Salicylate degradation	—	—	—	—	—	—	—	—	—	—
<i>p</i> -Aminosalicylate degradation	—	—	—	—	—	—	—	—	—	—
Acetamidase	—	—	—	—	—	—	—	—	—	—
Benzamidase	—	—	—	—	—	—	—	—	—	—
Urease	—	—	—	—	—	—	—	—	—	—
Isonicotinamidase	—	—	—	—	—	—	—	—	—	—
Salicylamidase	—	—	—	—	—	—	—	—	—	—
Allantoinase	—	—	—	—	—	—	—	—	—	—
Succinamidase	—	—	—	—	—	—	—	—	—	—
Growth at:										
28°C	+	+	+	+	+	+	+	+	+	+
37°C	+	+	+	+	+	+	+	+	+	+
45°C	—	—	—	—	—	—	—	—	—	—
52°C	—	—	—	—	—	—	—	—	—	—
Mannose as C source ^b	—	—	—	—	—	—	—	—	—	—
Sucrose as C source ^b	—	—	—	—	—	—	—	—	—	—
Propylene glycol as C source ^b	—	—	—	—	—	—	—	—	—	—
Galactose as C source ^b	—	—	—	—	—	—	—	—	—	—
Arabinose as C source ^b	—	—	—	—	—	—	—	—	—	—
Xylose as C source ^b	—	—	—	—	—	—	—	—	—	—
Rhamnose as C source ^b	—	—	—	—	—	—	—	—	—	—
Trehalose as C source ^b	—	—	—	—	—	—	—	—	—	—
Inositol as C source ^b	—	—	—	—	—	—	—	—	—	—
Mannitol as C source ^b	—	—	—	—	—	—	—	—	—	—
Sorbitol as C source ^b	—	—	—	—	—	—	—	—	—	—
Citrate as C source ^b	—	—	—	—	—	—	—	—	—	—
Succinate as C source ^b	—	—	—	—	—	—	—	—	—	—
Malate as C source ^b	—	—	—	—	—	—	—	—	—	—
Benzoate as C source ^c	—	—	—	—	—	—	—	—	—	—
Malonate as C source ^b	—	—	—	—	—	—	—	—	—	—
Fumarate as C source ^b	—	—	—	—	—	—	—	—	—	—
Acid from glucose	—	—	—	—	—	—	—	—	—	—
Acid from mannose	—	—	—	—	—	—	—	—	—	—
Glutamate as C and N sources	—	—	—	—	—	—	—	—	—	—
Serine as C and N sources	—	—	—	—	—	—	—	—	—	—
Glucosamine as C and N sources	—	—	—	—	—	—	—	—	—	—
Acetamide as C and N sources	—	—	—	—	—	—	—	—	—	—
Benzamide as C and N sources	—	—	—	—	—	—	—	—	—	—
Monoethanolamine as C and N sources	—	—	—	—	—	—	—	—	—	—
Trimethylene diamine as C and N sources	—	—	—	—	—	—	—	—	—	—
Benzamide as N source	—	—	—	—	—	—	—	—	—	—
Nitrite as N source	—	—	—	—	—	—	—	—	—	—

^a T-12109, *M. gordonae* ATCC 14470; 37004, *M. triviale* ATCC 19386; 38008, *M. terrae* W923; 37013, *M. triviale* ATCC 19387; 38013, *M. terrae* ATCC 15755; 09003, *M. nonchromogenicum* ATCC 19530.

^b Nitrogen from ammoniac.

^c TCH, Thiophene-2-carboxylic acid hydrazide.

done. After ultraviolet irradiation, ca. 17% of the surviving colonies appeared as scotochromogenic revertants, and S-type non-photochromogenic (pigmentless) mutants were isolated only at a rate of $1/10^4$ (2 per ca. 20,000 surviving colonies). The S-type non-photochromogenic mutants showed almost the same characters as

the parent strain, except for colony pigmentation.

Throughout our experiments, it was impossible to isolate non-photochromogenic mutants directly from scotochromogenic parent bacteria.

It was observed that the mutation from R-type photochromogens to R-type non-photo-

TABLE 2. *Change of catalase activity at various steps of mutation of M. gordonae T-12109 (ATCC 14470)*

Strain ^a	Foam height (mm) ^b
Parent	>130
P1	>130
P2	>130
P3	>130
P2-R1	>130
P2-R2	100
P2-R3	>130
P2-R1-N1	72
P2-R1-N2	53
P2-R1-N3	67
P2-R1-N4	52
P2-R1-N5	64
P2-R3-N1	>130
P2-R3-N2	>130
P2-R3-N3	>130
P2-R3-N4	59
P2-R3-N5	>130
R1	83
R2	52
R3	92
R1-P1	50
R1-P2	61
R2-P1	74

^a For designation of strains, refer to the text.^b Estimated by a semiquantitative method (9).

chromogens and the mutation from parent bacteria to R-type mutants frequently resulted in a decrease of catalase activity (Table 2).

The results of numerical classification, in which 88 characters were used, are shown in Fig. 2 as a dendrogram. Strain P2-R1 was included in the cluster of *M. gordonae*, whereas strains P2-R1-N4 and P2-R1-N7 were included in the cluster of the *M. nonchromogenicum* complex (*M. nonchromogenicum*, *M. terrae*, and *M. triviale*) (9). Strain P2-R1-N4 showed the highest matching coefficient to strain 37013 of *M. triviale* (97%), and strain P2-R1-N7 showed the highest one to 38013 of *M. terrae* (94%).

DISCUSSION

Comparison of pigments of *M. gordonae* and *M. scrofulaceum* from a genetic point of view. Previously, S. Tsukamura (11) reported that pigmentless (non-photochromogenic) mutants could be isolated from strains P5 and P6 of *M. scrofulaceum* after ultraviolet irradiation. In a recent study (unpublished), such mutants were isolated also from strains 12107 and 12108 of *M. scrofulaceum*. In contrast, non-photochromogenic mutants have not been isolated directly from strains of *M. gordonae*. Only photochromogenic mutants have been isolated from these strains. Photochromogenic mutants

have been isolated after ultraviolet irradiation not only from strain T-12109 but also from other two strains of *M. gordonae*, T-12353 (ATCC 23395) and T-12354 (ATCC 23397); non-photochromogenic mutants have not been isolated directly from parent, scotochromogenic bacteria (unpublished data).

These findings show that the genetic regulation of pigment formation of the scotochromogens *M. gordonae* and *M. scrofulaceum* is quite different. It is suggested that the pigments of *M. scrofulaceum* are formed in one way, which may be interrupted by the loss of one cistron. In contrast, the pigments of *M. gordonae* are formed in two ways: one in which light is required for pigment synthesis and another in which such a process is not required. It is also possible that the pigments are of two types: one formed in the dark and another formed only upon exposure to light. The colony pigmentation of the parent strain of *M. gordonae* is yellow or orange in the dark, and this is enhanced by exposure to light and becomes deep orange or

FIG. 2. Dendrogram showing the relationships among *M. gordonae*, *M. triviale*, *M. terrae*, *M. nonchromogenicum*, and mutants isolated from *M. gordonae* by ultraviolet irradiations. *M. gordonae*: T-12177, T-12180, T-12110, T-12109, T-12178, and T-12179. *M. triviale*: 37002, 37003, 37004, 37013 and 37005. *M. terrae*: 38001, 38007, 38008, 38004, 38006, and 38013. *M. nonchromogenicum*: 09013, 09016, 09023, 09033 and 09003. Mutant P2-R1 is included in the cluster of *M. gordonae*, and mutants P2-R1-N4 and P2-R1-N7 are included in the cluster of *M. nonchromogenicum* complex.

reddish orange. The pigments of photochromogenic mutants are reddish. It is suggested that the pigments formed in the dark are different from those formed upon exposure to light.

Complex mutation. The phenomenon in which a mutation accompanies other mutations in the same cell is called "complex mutation." Complex mutations have been studied extensively in wheat (12), and the presence of such a complex mutation in mycobacteria has been reported by Tsukamura (5, 6). This phenomenon was observed in the present study. The mutation from R-type photochromogens to R-type non-photochromogens was sometimes accompanied by loss of the ability to utilize certain carbon sources (Table 1).

Furthermore, it was also observed that the mutation from R-type photochromogens to R-type non-photochromogens and the mutation from parent bacteria to R-type mutants frequently caused a decrease of catalase activity.

It is noteworthy that complex mutation did not occur in all cases but only in specified ones. For example, the mutation from S-type scotochromogens to S-type photochromogens and the mutation from S-type photochromogens to S-type non-photochromogens did not produce simultaneous changes of characters as observed in the present study, whereas the mutation from R-type photochromogens to R-type non-photochromogens (from P-R to P-R-N) caused simultaneous alteration of other characters.

Definition of species. There is, at present, no decisive definition of the bacterial species concept. It may be defined, from viewpoints of numerical taxonomy, as a well-defined cluster (3) or, from viewpoints of immunology, as a group of organisms which share the same antigens (4). According to the former definition, strains P2-R1-N4 and P2-R1-N7 are included in the cluster of the *M. nonchromogenicum* complex (*M. nonchromogenicum*, *M. terrae*, and *M. triviale*) (9) and, therefore, are regarded as members of the complex. Strains P2-R1-N4 and P2-R1-N7 show matching coefficients of 97 and 94% to strain 37013 (ATCC 19387) of *M. triviale* and to strain 38013 (ATCC 15755) of *M. terrae*, respectively. On the other hand, they show coefficients of 82 and 90% to strain T-12109 (ATCC 14470) of *M. gordonae*, from which they have been derived. This finding raises the question of whether *M. triviale*, *M. terrae*, and/or *M. nonchromogenicum* can be accurately differentiated from *M. gordonae*. However, a change of the species concept due to mutational studies as done here may result in great confusion. To avoid this, "species" should be defined as a well-

recognizable cluster which is formed by "natural selection." The organisms of the *M. nonchromogenicum* complex belong to such a species.

In the present study, strains phenetically indistinguishable from *M. triviale* and/or *M. terrae* were isolated from the type strain of *M. gordonae*. However, mutants which show, like *M. nonchromogenicum*, nicotinamidase, pyrazinamidase, and β -galactosidase activities have not yet been obtained.

Strains P2, P2-R1, P2-R1-N4, and P2-R1-N7 have been deposited in the American Type Culture Collection, Rockville, Md., as ATCC 33005, ATCC 33006, ATCC 33007, and ATCC 33008, respectively.

REPRINT REQUESTS

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