1969

Phage Induced Changes in Enzymatic Activity of Lysogenic Mycobacterium Smegmatis

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by

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A Thesis Submitted to the Faculty of the Graduate School of Loyola University in Partial Fulfillment of the Requirements for the Degree of Master of Science

June

1969

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INTRODUCTION

Lysogeny is the hereditary property of a given bacterium to produce bacteriophage without further infection by external particles. Lysogenic bacteria carry the phage genome as an integral part of their chromosome which they can transmit to their progeny (27). This additional genetic material can alter the properties of the host. The first evidence for this was the discovery that diphtheria phage β was responsible for toxin production in *Corynebacterium diphtheriae*. Freeman in 1951 isolated a phage from *C. diphtheriae* and demonstrated that only diphtheria bacilli lysogenic for phage β or closely related bacteriophage are toxigenic (13). He attributed toxin production to transduction, but evidence accumulated indicating that the phage genome itself, and not transfer of a chromosomal segment from an earlier host, caused the change (3, 15 through 18). Groman (16) used the phage isolated by Freeman, but passed it through non-toxigenic strains before lysogenizing another set of non-toxigenic strains. He confirmed that only lysogens were capable of producing toxin, but dispelled the idea that it was due to transduction (17,18). Groman used the term conversion (16) to avoid repeating the phrase "the change from the non-toxigenic to the toxigenic state" (18), but the term "conversion

by bacteriophage" subsequently was used to refer to all modifications of the host resulting from infection by phage (4) and the phage that caused the change was termed a "converting phage" (4). In a more restricted sense, phage conversion "appeared to be the result of the addition of information to the host genome" (18), in the case of diphtheria toxin production the addition of a nucleotide base sequence necessary to code for the protein toxin (11). In *lysogenic conversion* the hereditary modification of the host chromosome requires integration of the phage genome. Barksdale *et al.* (3) used ultraviolet irradiation to induce the lytic cycle of phage in lysogenic strains of *C. diphtheriae* They showed that lytic propagation of the phage could lead to higher levels of toxin production than was found in lysogenic strains. They concluded that the phage genome was solely responsible for toxin production in toxinogenic diphtheria strains (4). Miller *et al.* (29) have shown, on the other hand, that the enzymatic machinery of the host plays a role in toxin production, and that in bacteria which had defective cytochrome oxidase metabolism, toxin production was greatly diminished. However, the phage released from lysogenic strains with defective metabolism produced normal levels of toxin when used to infect diphtheria bacilli with intact enzymatic apparatus.

Other examples of lysogenic conversion are: erythrogenic toxin production in group A streptococci (42), changes of phage type in *Staphylococcus aureus* (6,31), and serotype changes in *Salmonella* (19,35-37).

With respect to *Mycobacterium*, Bönicke reported loss of α-nicotinic acid oxidase activity and gain of malachite green reductase activity in *Mycobacterium smegmatis* SN2 lysogenized with mycobacteriophage B1 (9). Jones and White found changes in nitrate reduction, Tween-80 hydrolysis and colonial morphology in *M. smegmatis* ATCC 607 following lysogenization with phage D26 or phage B4 (20). Juhasz observed decreased growth rate, altered colonial morphology, and inability to synthesize thiamine in *M. phlei* F89 lysogenized with phage B2h (22). Mankiewicz *et al.* found several changes in ATCC 607 lysogenized with phage Roy (28). They reported decrease in growth rate, paraffin utilization, acetamidase, catalase and nitrate reductase activity, and in the ability to "transform" ammonium ferrocitrate.

The enzymes chosen for the present study were amidases, phenolphthalein sulfatase, nitrate reductase, and malachite green reductase. The amide series introduced by Bönicke (7,8) consists of ten amides (Fig. 1), the hydrolysis of which is of taxonomical significance (8,23). These are acetamide, benzamide, urea, isonicotinamide, nicotinamide, pyrazinamide, salicylamide, allantoin, succinamide, and malonamide.

Nitrate reductase reduces nitrate to nitrite (38).

$$NO_3^- \xrightarrow{\text{Nitrate Reduct.}} NO_2^- + 1/2\ O_2$$

Phenolphthalein sulfatase hydrolyses the phenolphthalein disulfate to free phenolphthalein (41).

$$\text{Phenolphthalein disulfate} \xrightarrow[\text{Phenolphthalein Sulfatase}]{+2H_2O} \text{Phenolphthalein} \quad \text{3,3-Bis(p-hydroxyphenyl) phthalide}$$

Phenolphthalein is an indicator since at pH ∿ 8 the red dimetallic ion is formed. This makes its presence easily detectable.

The choice of these enzymatic activities for study was based on the following considerations:

1. There are easily reproducible tests available for studying these enzymes (7,8,38,41).

2. The amidase, phenolphthalein sulfatase, and nitrate reductase activities of mycobacteria have previously been extensively studied for taxonomic purposes (7,8,23,38). Changes in well established patterns may therefore have taxonomic implications.

3. The ability to reduce nitrate to nitrite (20,28) and malachite green to its leucoform (9) have been previously compared for wild and lysogenic mycobacteria.

In the course of this study *Mycobacterium smegmatis* strains SN2, SN10 and SN14 have been lysogenized with mycobacteriophage B_1 (21) and their enzymatic activity was determined before and after lysogenization.

FIGURE I AMIDES OF BÖNICKE'S AMIDE SERIES(7).

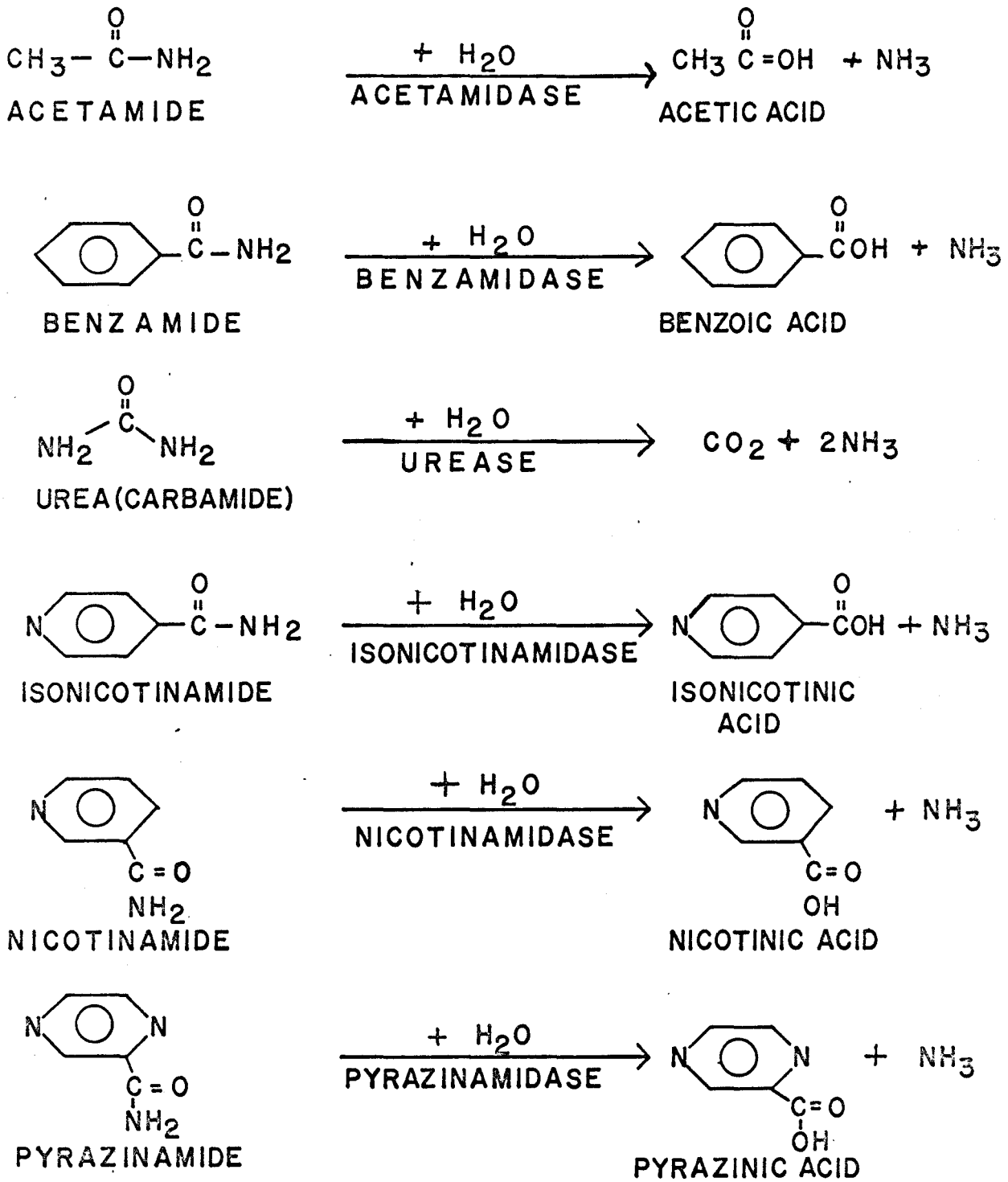
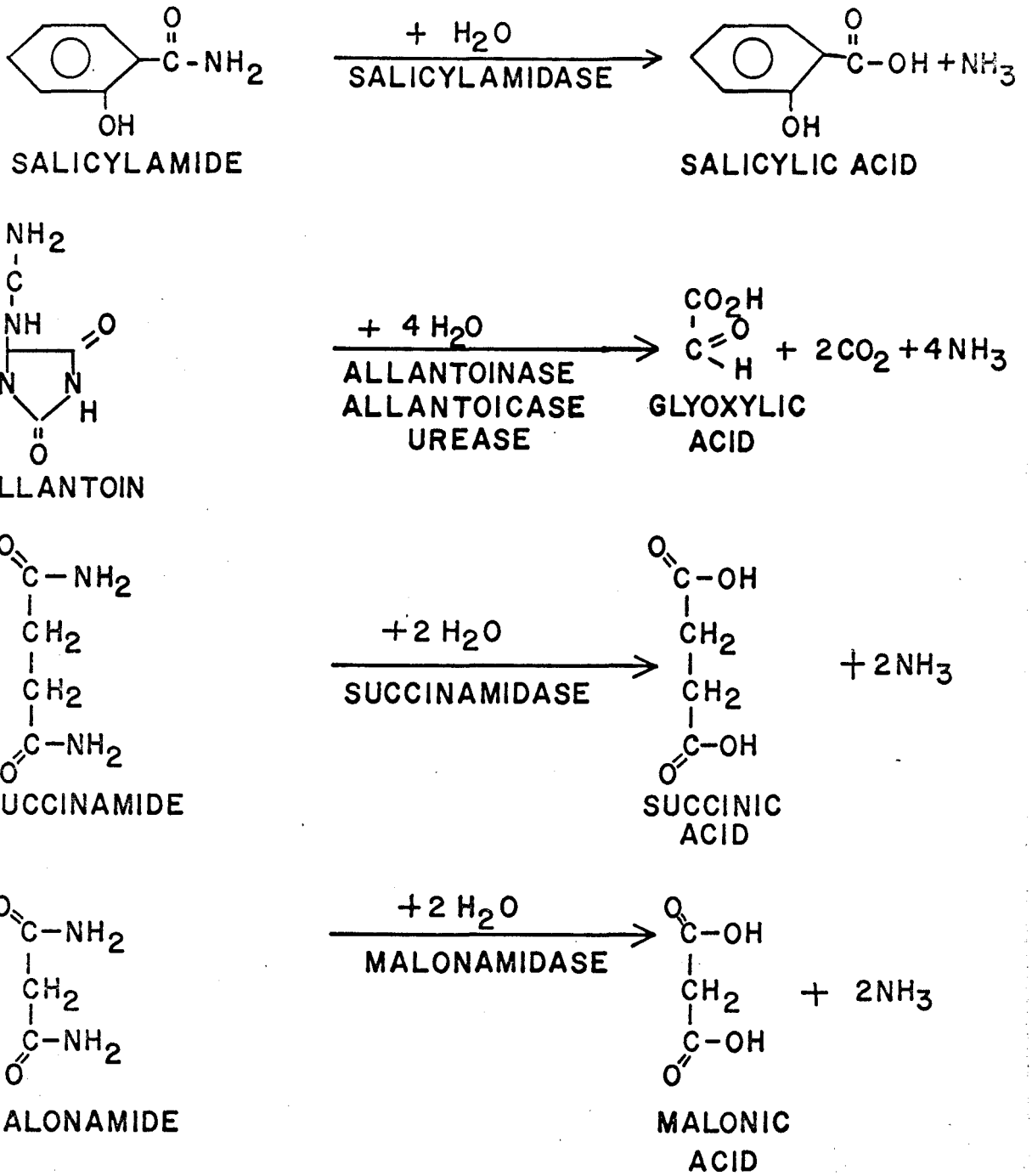


FIGURE 1 (CONT.)



MATERIALS AND METHODS

a. *Bacterial strains.* *Mycobacterium smegmatis* strains SN2, SN10, and SN14 of the Borstel Collection, Borstel, West Germany, served as hosts for this study. The original designation of SN10 was ATCC 607, considered for a long time to be an original Koch strain of *Bacillus tuberculosis* (2).

b. *Phage strain.* Mycobacteriophage smegmatis B1 (21) harvested from its propagating host *Mycobacterium smegmatis* SN2 was used to infect and lysogenize all *M. smegmatis* strains.

c. *Media.* Bacterial cultures were maintained on Loewenstein-Jensen slants and plates (courtesy of Hines V.A. Hospital, Hines, Ill.). Phage cultures were kept and phage dilutions were made in heart infusion broth (Difco, Detroit, Mich.). Lysogenization experiments were performed on nutrient agar (Difco, 1.5%) plates; the agar overlay was prepared from nutrient broth (Difco) by the addition of 0.7% Bacto-agar (Difco). Nutrient agar (Difco, 1.5%) was used for the purification of wild strains and lysogens.

d. *Phage propagation and counting of phage particles.* The phage was assayed by the plaque count method of Gratia (1) using the modification of Froman *et al.* (14). Indicator *Mycobacterium smegmatis* SN2 organisms were inoculated into melted and cooled agar and then poured on top of a nutrient agar base plate. After solidification of the top agar layer, 0.05 ml of ten-fold serial dilutions of the phage were dropped in duplicate, one sample on each half of a plate. After 5 days incubation at 37 C, the

plates were assayed. The titer was determined as the number of individual plaques multiplied by the dilution and extrapolated for 1.0 ml of the undiluted phage suspension. The bacteriophage suspension was subsequently diluted to contain 10^9 PFU (plaque forming units) per ml.

e. *Purification of parent strains.* Parent strains were purified by six serial single colony transfers on nutrient agar.

f. *Preparation of lysogens.* A suspension prepared from the sixth passage of the parental strain was inoculated into top agar and infected with 0.05 ml of phage using the agar overlay method described earlier. Colonies which appeared in the lytic zones were purified by six serial single colony transfers on nutrient agar. They were then tested for lysogeny on the basis of two criteria: (a) their ability to produce plaques when plated on a phage-susceptible parental *M. smegmatis* strain and (b) their immunity to superinfection by phage B1 (1).

g. *Preparation of suspensions for enzymatic studies.* Those strains which conformed to the two previously mentioned criteria of lysogeny, and, for control purposes, their parent strains, were heavily inoculated on Loewenstein-Jensen plates and incubated for 12-15 days at 37 C. The bacteria were then harvested by using sterile scalpel blades to scrape the growth off the surface of the plate, taking special care to exclude any medium. They were weighed after being suspended in tared tubes containing physiological saline. They were washed by centrifugation for 20 min at

3000 rev/min (International Equipment Company [Boston, Mass.], size two, clinical centrifuge) and resuspended in pH 7.2 M/15 sodium-potassium phosphate buffer to give 10.0 mg dry weight per ml. This suspension was then used for all the enzymatic tests.

h. *Amidase test.* Bönicke's amide series (7,8) was employed to test for amidase activity. One ml buffered suspension of a given strain was mixed with 1.0 ml of 0.00164 M solution of each of the following amides: acetamide, urea, isonicotinamide, benzamide, nicotinamide, salicylamide, succinamide, malonamide, pyrazinamide, and allantoin. These were presterilized by heating at 100 C in a water bath for 30 min, except for urea which was filtered through a 0.45 μ membrane filter, type HAWP, 4700 (Millipore Corp., Bedford, Mass.). Each amide-bacterium mixture was incubated for 22 hr at 37 C. Liberation of ammonia resulting from the hydrolysis of a given amide was detected by the phenol-hypochlorite method described by Russell (32). One-tenth ml of 0.003 M $MnSO_4 \cdot H_2O$, 1.0 ml of phenolate reagent, and 0.5 ml of KClO, was added to each tube, in the given order, with a minimum delay between the phenolate and the hypochlorite. Since this method, originally reported by Berthelot (5), does not produce a stable colored complex, time is critical when determining the concentration of the complex (40). Color development was obtained by heating the tubes in a water bath at 100 C for 15 min. Five min were allowed for cooling at room temperature. The tubes were then estimated visually immediately by

comparison with standards containing known concentrations of NH_3, as described by Bönicke (7,8) and Juhlin (23). Subsequently, parallel tests were measured spectrophotometrically using a Gilford model 2000 spectrophotometer to measure the absorbance of the complex at 550 nm after removal of bacteria by centrifugation at 2500-3000 rev/min. The standards used for these tests were 0, 2, 4, 6, and 8 µg NH_3/ml added as $(NH_4)_2SO_4$. The controls employed were amide solutions without bacteria, and bacteria without amides. Two ml of physiological saline plus reagents were employed as the blank for the spectrophotometric determination. The intensity of color in the visual estimates was expressed by a 0 to +++ scale which is based on the µg of NH_3 produced by hydrolysis of a given amide per ml.

Reagents for the amidase test and their preparation. The phenolate was prepared by suspending 25 g of phenol (ACS reagent, J. T. Baker Chemicals, Phillipsburg, N.J.) in 10 ml distilled water, adding 54 ml of 5 N NaOH and adjusting the solution to a final volume of 100.0 ml. This solution had to be prepared freshly. The hypochlorite (Eau d'Javel, Germany) was provided by Dr. R. Bönicke, Research Institute for Experimental Biology and Medicine, Borstel, West Germany. $MnSO_4 \cdot H_2O$ (ACS reagent), benzamide, nicotinamide, salicylamide, succinamide, malonamide, and $(NH_4)_2SO_4$ (ACS reagent) were all obtained from J. T. Baker Chemicals; acetamied, urea, and isonicotinamide, from Sigma Chemical Co., St. Louis, Mo.; allantoin and pyrazinamide from Dr. R. Bönicke. The former was a product of Fluka A.G., Buchs S.G., Switzerland, and the latter of Krugman and Co., Hamburg, Germany (7).

i. *Nitrate reductase test.* Virtanen's nitrate reductase test for mycobacteria (38) was used to detect the reduction of nitrate to nitrite. A 0.04 ml sample of a suspension containing 0.4 mg dry weight of bacteria was inoculated into nitrate broth (Difco) and incubated at 37 C for 2 hr. The color reaction of Shinn (33) was used to detect the amount of nitrite produced. After incubation, 0.1 ml of a 1:1 dilution of concentrated HCl was added to each tube and mixed in a vortex mixer for approximately 30 sec. A 0.1 ml sample of aqueous 0.2% sulfanilamide and 0.1 ml of aqueous 0.01% N-1-naphthylethylenediamine were then added to each tube and mixed on a vortex mixer. Color developed to its maximum after approximately five min at room temperature.

The color reaction based on the formation of a diazocomplex can be measured either by visual comparison of the experimental tubes with standards of known concentration (23,38) which is more accurate than comparison with arbitrary color standards (23,25,38), or colorimetrically (30). The color reaction indicating reduction of nitrate to nitrite is based on the formation of a red azo compound. This involves, first, the reaction in acid solution of a primary amine, such as sulfanilic acid or sulfanilamide,with nitrite to form a diazonium salt. The latter is then coupled to an aromatic amine to yield the red azo dye. The concentration of the azo dye can be determined on a Klett-Summerson colorimeter employing a 540 nm (green) filter. Using the quantity of reagents specified, 10 Klett units equal 1 mμmole of nitrite. This was calibrated by preparing a 35 mμmolar aqueous

solution of $NaNO_2$ (69.0 mg/l) to see if this produced a reading of 350 Klett units. The relationship between µg of nitrite/ml as measured by the standards and mµmoles/ml as determined colorimetrically is given in figure 2 so that a direct comparison of the methods can be made.

Initially the visual method of comparison with standards was used for all strains. Subsequently representative strains of each group were selected for the colorimetric determination employing a Klett-Summerson photoelectric colorimeter. The standards prepared for comparison were 0, 0.1, 1.0, 5.0, 10.0, and 50.0 µg nitrite/ml. Sterile nitrate broth served as the control and was used as the blank, after addition of reagents for the colorimetric determination. Negative results were tested for technical error by addition of zinc dust (38) which reduced nitrate to nitrite chemically and produced a positive color reaction if all necessary reagents had been added.

Reagents for nitrate reductase test. Sulfanilamide was obtained from Eastman Organic Chemicals, Rochester, N.Y.; N-1-naphthylethylenediamine from Mann Research Laboratories, New York, N.Y. $NaNO_2$ (ACS reagent) was obtained from Merck and Co., Rahway, N.J. The standard nitrite solution was prepared by adding 69.0 mg $NaNO_2$ to 1 l. distilled water. This gave an absorbance of 350 Klett units (30).

j. *Phenolphthalein sulfatase test.* Wayne's modification (39) of the arylsulfatase test of Whitehead *et al.* (41) was employed for this test.

FIG.2 RELATIONSHIP BETWEEN KLETT READING AND NITRITE μg/ml

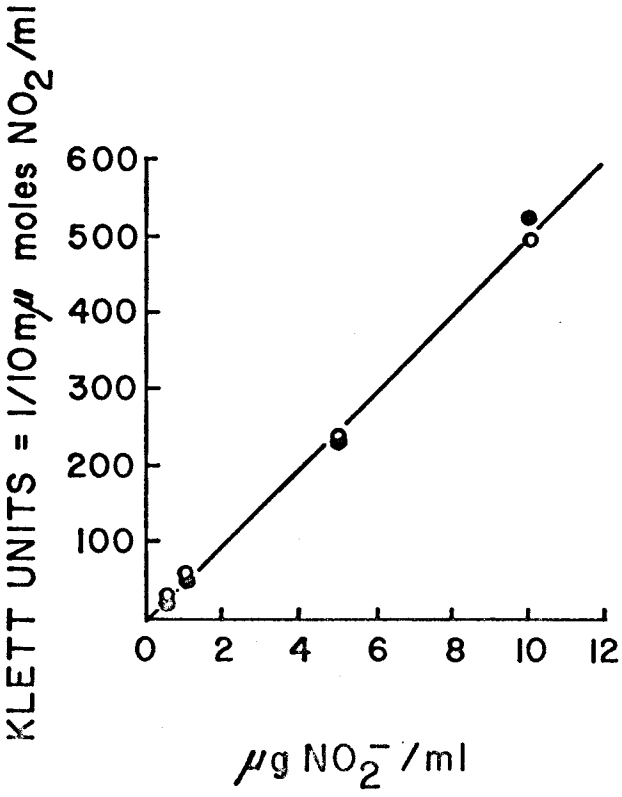
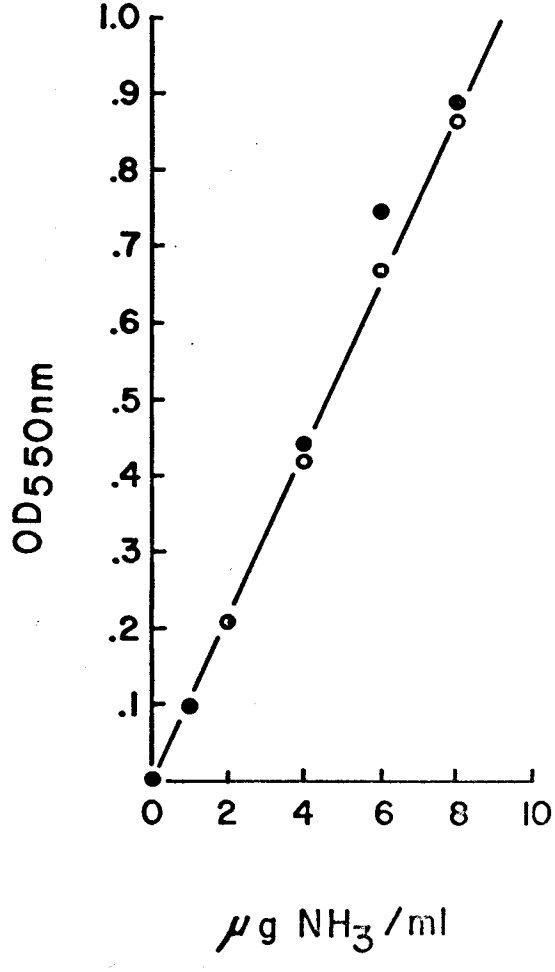


FIG.3 STANDARD CURVE FOR AMIDASE TESTS



Dubos oleic acid agar (Difco) containing 650 mg of phenolphthalein disulfate, tripotassium salt per liter was distributed in 2.0 ml amounts into tubes. Tubes inoculated with a suspension containing 0.4 mg dry weight of bacteria were subsequently incubated at 37 C for 3, 14, and 21 day periods. The quantity of phenolphthalein liberated by hydrolysis of the substrate was detected by the addition of 0.2 ml of 2 N Na_2CO_3. The tubes were kept at 4 C to permit diffusion of the color through the medium and then visually compared with standards containing known amounts of phenolphthalein. The standards proposed by Sommers and Russell (34) were employed. The concentration of phenolphthalein in the standards was as follows: 0, 2, 6, 16, and 40 µg/ml. A strain of *Mycobacterium fortuitum* SN203, from the Borstel Collection, was used as the positive control for the 3 day test. Uninoculated tubes served as negative controls in all tests.

Reagents for phenolphthalein sulfatase test. Phenolphthalein disulfate tripotassium salt was obtained from Eastman Organic Chemicals, Rochester, N.Y.; phenolphthalein from Merck and Co., Inc., Rahway, N.J., Na_2CO_3 (ACS reagent) from J. T. Baker Chemicals, Phillipsburg, N.J.

Preparation of standards for phenolphthalein sulfatase test (26).

	Distilled H_2O	Conc.	Visual Reading
1. 1 ml stock solution*	to 50 ml	200 µg/ml	
2. 5 ml of tube #1	to 25 ml	40 µg/ml	4+
3. 2 ml of tube #1	to 25 ml	16 µg/ml	3+
4. 1.5 ml of tube #1	to 50 ml	6 µg/ml	2+
5. 0.5 ml of tube #1	to 50 ml	2 µg/ml	1+

* 0.1 g phenolphthalein in 10.0 ml of 95% ethanol.

Solutions lacking Na_2CO_3 were stored at 4-10 C for several months. After addition of 2.0 ml of 2 N Na_2CO_3 the solutions were stable for 2-4 weeks at 4-10 C.

k. *Malachite green reductase test.* Malachite green reduction can be detected by the ability of an organism to decolorize Loewenstein-Jensen medium when propagated on the latter (9). The range of the indicator made it unlikely that the reaction was a reversible reduction of the malachite green due to change in pH. Therefore, an additional test was performed in liquid medium in order to study the pH changes, if any, caused by growth. Liquid Loewenstein-Jensen medium was prepared in which the egg constituent of the Loewenstein-Jensen medium was replaced by an equal volume of pH 7.0 M/15 sodium potassium phosphate buffer. The solution obtained was autoclaved at 120 C for 30 min instead of being inspissated and was distributed in 2.0 ml amounts into tubes. A sample containing 0.4 mg of each strain was inoculated into duplicate sets of tubes and incubated at 37 C until decoloration of the medium occurred or for 14 days. Uninoculated tubes of the liquid medium were used as controls. After decolorization of the medium, bacteria were removed by filtration using a 0.45 μ Millipore filter type HAWP, 4700 and the filtrate was tested for pH indicator properties by adjusting its pH with 2 N HCl and 2 N NaOH ranging from a pH of 0 to a pH of 10.

RESULTS

While all four of the *Mycobacterium smegmatis* SN2 B1 [hereafter referred to as SN2 (B1)] lysogens resulted from a single lysogenic event, the *M. smegmatis* SN10 (B1) [hereafter referred to as SN10 (B1)] and *M. smegmatis* SN14 (B1) [hereafter referred to as SN14 (B1)] lysogens used for this study were obtained in two different lysogenic events. For example, SN10 (B1) 2 and SN10 (B1) II were isolated in different experiments. This is indicated in the tables by the use of arabic and roman numerals.

Amidase activity. The results of the amidase tests are presented in Tables 1-4. Triplicate tests were made whenever duplicate tests were not in good agreement. All strains were tested by comparison with standards of known concentration (Tables 1-3). Selected strains were also retested spectrophotometrically, along with their parental strains (Table 4). For this purpose two representative lysogens were chosen from each set. Tables 1, 3, and 4 show that the most significant change, a change of > 3 μg NH_3/ml, that was noticeable in strains SN2 and SN14 upon lysogeny, was a drastic decrease in their salicylamidase activity. This change could not be observed in SN10 lysogens, since in contrast to wild SN2 and SN14, SN10 does not possess appreciable salicylamidase activity (Tables 2 and 4). The only other amidase which was affected by lysogenization was isonicotinamidase in SN2 (B1) 4 (Tables 1 and 4). Readings by the visual comparison method indicated that varying degrees of reduction in benzamidase and isonicotinamidase activity occurred in several strains of SN2 (B1) and SN14 (B1)

lysogens, but this was not tested for all strains using the more precise spectrophotometric assay (Table 4). The difference in salicylamidase activity as measured spectrophotometrically was an average of 3.5 μg NH_3/ml produced by SN2 as compared to a maximum of 0.5 μg NH_3/ml produced by the SN2 (B1) lysogens, and 4.0 μg NH_3/ml produced by SN14 as compared to a maximum of 0.5 μg NH_3/ml produced by SN14 (B1) lysogens. This represents a difference of nearly 0.400 OD_{550} corresponding to a difference of + compared with 0 in the evaluation system of Bönicke (7,8) as summarized in Table 5. The difference in isonicotinamidase activity between SN2 and SN2 (B1) 4 is not as significant, with the former producing 6.0 μg NH_3/ml as compared to 3.0 produced by the latter. This however corresponds to a relative difference of ++ to +. Differences between the two methods are summarized by comparison of Tables 5 and 6. The values obtained by the spectrophotometric method are generally lower than that obtained by visual comparison to standards. The range of NH_3 produced per ml was within the Beer's law region as demonstrated by the linear relationship between OD_{550} and μg NH_3/ml (Figure 3).

Nitrate reductase activity. The results of the nitrate reductase tests are presented in Table 7. In all lysogens of SN2 and SN14 a decrease in nitrate reductase activity was observed when compared to the parental strains. This was more striking in SN14 (B1) than in SN2 (B1) strains because wild SN2 produced only 4 μg nitrate/ml while SN14 produced 10 μg nitrite/ml.

In SN2 (B1) strains the decrease was from 4 µg nitrite/ml produced by wild SN2 to undetectable quantities in the lysogens except for SN2 (B1) 2 which showed an activity of 1 µg nitrite/ml. With the exception of SN14 (B1) 3 all SN14 lysogens appeared to have lost the ability to reduce nitrate to nitrite.

In SN10 (B1) lysogens on the other hand, varying degrees of nitrate reduction could be shown. One strain, SN10 (B1) II retained the same nitrate reductase activity as wild SN10, which produced 10 µg nitrite/ml; two strains, SN10 (B1) 2 and SN10 (B1) I, showed a somewhat reduced activity, while one strain, SN10 (B1) 1, exhibited a substantial increase in activity, producing over 50 µg nitrite/ml instead of 10 µg nitrite/ml of wild SN10. These results were confirmed by colorimetric readings of the nitrate reductase activity of representative strains (Table 8).

Phenolphthalein sulfatase activity. The results of the phenolphthalein sulfatase tests are presented in Table 9. The three day test used in the identification of rapidly growing mycobacteria (39), was negative for all strains except the positive control, *M. fortuitum*, and was not repeated. The twenty-one day test, in contrast, was positive to essentially the same degree for all strains. Differences were observed only in the fourteen day test which was performed in triplicate. Three out of the four SN2 (B1) lysogens exhibited only trace activity as compared to the parent. Only SN2 (B1) 1 was similar to wild SN2 and produced 6 µg phenolphthalein/ml

as compared to 8 µg/ml of the latter. All of the SN14 lysogens exhibited decreased phenolphthalein sulfatase activity as compared to the parent SN14. SN14 (B1) 1 and SN14 (B1) 3 displayed no detectable activity, but SN14 (B1) I and SN14 (B1) 2 showed an activity resulting in the production of 4 µg phenolphthalein/ml and 6 µg/ml respectively.

SN10 lysogens gave varying results. SN10 (B1) I showed an increased activity, production of 20 µg phenolphthalein/ml, as compared to only 5 µg phenolphthalein/ml produced by wild SN10. SN10 (B1) II, in contrast, displayed no detectable activity, while SN10 (B1) 2 behaved as the wild SN10.

Malachite green reduction tests. The results of the malachite green reduction tests are presented in Table 10. With the exception of SN10 (B1) 1, all lysogens decolorized malachite green when grown on Loewenstein-Jensen medium. Furthermore, all strains, parental and lysogens, decolorized malachite green in a medium in which egg was replaced by a pH 7.0 phosphate buffer. Decolorization occurred in all tubes except the uninoculated control after 7-10 days (Table 10). Growth of all strains, including the parents, seemed retarded. Visible growth could be observed after 7 days as compared to 3 days on solid medium (Table 10). The sterile filtrates of these tubes of liquid medium revealed that the malachite green had been either degraded or complexed since no pH indicator properties could be detected.

Colonial morphology and appearance of growth in broth. The growth of the lysogens on nutrient agar, Loewenstein-Jensen medium, and in nutrient broth differred substantially from that of the parent strains in most cases. Colonies of the SN2 lysogens were smaller and more mucoid than parent SN2 on both Loewenstein-Jensen medium and on nutrient agar. They also had a smooth glistening appearance on these media as compared to the rough colonies of the parent. In broth, SN2 lysogens formed thin films on the surface of the broth while the parent formed a granular suspension which settled rapidly to the bottom of the flask. Colonies of the SN10 lysogens were of the same size as wild SN10 but were more mucoid on solid media. SN10 lysogens did not form films in liquid media, but rather formed fine threads which when shaken settled to the bottom. The SN14 lysogens were smaller and more mucoid than parent SN14; furthermore, they were less pigmented. While the parent SN14 colonies were tan, the lysogens were grey resembling wild SN2 or SN10. In broth, SN14 lysogens produced the same granular growth as the parent.

All lysogens examined grew deep into Loewenstein-Jensen medium which made removal difficult. Parent SN2, SN10, and SN14 strains, in contrast, grew only at the surface and could easily be removed with a wire loop.

TABLE 1

Amidase Activity of SN2 and Its Lysogenic Derivatives by Visual Comparison to Standards

Amides	SN2	SN2(B1)1	SN2(B1)2	SN2(B1)3	SN2(B1)4
Acetamide	8,5,5.5 6	4.5,8,4 6	8,6 7	4,5,7 5	4.5,3 4
Benzamide	7,5,4.5 6	4,3,2.5 3	4,3 4	3,2,1 2	4,3.5 4
Urea	8,8,5.5 7	9,6,7 7	10,8 9	7,6,7.5 7	7,7 7
Isonicotinamide	7,8,5.5 7	4,2,3.5 3	8,4 6	2.5,4,2.5 3	2.5,1 2
Nicotinamide	8,8,8 8	6,6,6 6	8,7 8	6.5,3.5,4 4	6,6 6
Pyrazinamide	-,6.5,6.5 6	-,-,5.5 6	-,6 6	-,-,5.5 6	5.5,6 6
Salicylamide	4.5,2.5,4 4	1,0,1.5 1	1.5,1.5 2	2,1,0.5 1	1,0.5,0 1
Allantoin	2,1.5,0 1	1,0,0.5 0	1.5,0.5 1	0,0,0 0	0.5,1 1
Succinamide	8,8.5,7.5 8	9,8,7 8	9,7.5 8	7.5,6,7 7	7.5,8 8
Malonamide	2,2.5,1.5 2	1,2,0 1	1,0 0	0.5,1,2 1	0,0 0

Results are expressed in μg NH_3 produced per ml of the bacterium-amide mixture after incubation for 22 hr at 37 C. Pyrazinamide was not available for the first series of experiments. Preliminary experimentation showed, however, no differences between the pyrazinamidase activity of lysogens and their parents. The mean value rounded off to the nearest μg is shown under the actual data for each amide of each strain.

TABLE 2

Amidase Activity of SN10 and Its Lysogenic Derivatives by Visual Comparison to Standards

Substrate	SN10	SN10(B1)1	SN10(B1)2	SN10(B1)I	SN10(B1)II
Acetamide	3,4,6 4	7.5,4.5,6 6	4.5,4.5 4	4,5.5 5	4.5,6 5
Benzamide	6,7,6 6	5.5,4,4 4	4.5,4 4	3.5,5.5 4	3.5,5.5 4
Urea	7,8,7 7	7,8,10 8	7,8 8	7.5,10 9	7,8 8
Isonicotinamide	6.5,6,6.5 6	3.5,3.5,5 4	3,3 3	4,8 6	4,7.5 6
Nicotinamide	6,7,6 6	6,4,7 6	5.5,3.5 4	5.5,6 6	5.5,7.5 6
Pyrazinamide	5.5,5.5 6	5.5,7 6	5.5,5.5 6	4.5,6 5	6,7 6
Salicylamide	0.5,1,1 1	0,0,1 0	0,0 0	0,0 0	0,1 0
Allantoin	2,2,0 1	1,0,0 0	0,0 0	0,0 0	0,0 0
Succinamide	6.5,7,6.5 7	7.5,5.5,8 7	7,5.5 6	6.5,6 6	6.5,8 7
Malonamide	2,0,1 1	0.5,0,1 0	0,0 0	0,0 0	0,0 0

Results are expressed in μg NH_3 produced per ml of bacterium-amide mixture after incubation for 22 hr at 37 C. The mean value rounded off to the nearest μg is shown under the actual data for each amide of each strain.

TABLE 3

Amidase Activity of SN14 and Its Lysogenic Derivatives by Visual Comparison to Standards

Substrate	SN14	SN14(B1)1	SN14(B1)2	SN14(B1)3	SN14(B1)I
Acetamide	5,8,8 7	4.5,7 6	3,2 2	4,5,5 5	3,4 4
Benzamide	7,7,6 7	4.5,5 5	3,2 2	3,2,4.5 3	4,5 4
Urea	7,8,7.5 8	7,7 7	6.5,4 5	7,5.5,7 6	7,8 8
Isonicotinamide	7,7,7 7	4,3.5 4	2,2 2	2,5,6 4	3.5,5 4
Nicotinamide	7,8,7.5 8	4,6 5	3.5,3 3	5,3.5,6 5	4.5,7 6
Pyrazinamide	-,6,6 6	6,5.5 6	6.3.5 5	6,5.5,6 6	5,7 6
Salicylamide	5.5,4,2 4	0,0 0	1.5,0 0.7	2,1,0.5 1	0,1 0
Allantoin	1,0,0 0	0,0 0	0,0 0	0,0 0	0,0 0
Succinamide	6,8,7.5 7	6,7 6	6.5,4 6	5,6.5,10 7	4,8 6
Malonamide	1,3,0.5 2	0,0 0	0,0 0	0,0 0	0,0 0

Results are expressed in μg NH_3 produced per ml of bacterium-amide mixture after incubation for 22 hr at 37 C. Pyrazinamide was not available for the first series of experiments. The mean values rounded off to the nearest μg are shown under the actual data for each amide of each strain.

TABLE 4

Amidase Activity of Representative Strains
Spectrophotometrically Determined

Substrate		SN2	SN2(B1)3	SN2(B1)4
Acetamide	OD	.643, .690	.480, .413	.574, .609
	µg	6.0	4.0	5.5
Benzamide	OD	.502, .475	.320, .384	.347, .390
	µg	4.5	3.5	3.5
Urea	OD	.677, .652	.764, .741	.831, .708
	µg	6.0	7.0	7.5
Isonicotinamide	OD	.643, .735	.637, .600	.399, .220
	µg	6.0	4.0	3.0
Nicotinamide	OD	.712, .795	.527, .550	.572, .478
	µg	6.0	5.0	5.0
Pyrazinamide	OD	.546, .551	.610, .564	.570, .430
	µg	5.0	5.0	4.5
Salicylamide	OD	.367, .380	.066, .070	.000, .013
	µg	3.5	0.5	0.0
Allantoin	OD	.082, .075	.072, .080	.000, .000
	µg	1.0	1.0	0.0
Succinamide	OD	.787, .770	.846, .831	.866, .900
	µg	7.0	7.5	8.0
Malonamide	OD	.223, .180	.060, .072	.000, .020
	µg	1.5	0.0	0.0

Top numbers in each column represent the actual $O.D._{550}$ read in duplicate experiments. Bottom numbers represent average NH_3 production in µg/ml (for the exact relationship between µg/ml and $O.D._{550}$, see Fig. 3). Figures are rounded off to the nearest 0.5 µg which is the limit of sensitivity of the Berthelot reaction (40). To convert OD_{550} into µg NH_3/ml, Beer's law was applied:

$OD_{550} = \varepsilon CL$ L = 1.0 cm for the Gilford-2000 $\frac{OD_{550}}{\varepsilon \times 1.0} = C$

ε = .110 ml/µg (from Fig. 3) $\frac{OD_{550}}{.110} = C$ in µg/ml

TABLE 4a

Amidase Activity of Representative Strains
Spectrophotometrically Determined

Substrate		SN10	SN10(B1)1	SN10(B1)I
Acetamide	OD	.572, .482	.464, .490	.452, .334
	µg	5.0	4.0	4.0
Benzamide	OD	.552, .468	.462, .294	.447, .512
	µg	4.5	4.5	4.0
Urea	OD	.825, .711	.847, 1761	.862, .776
	µg	7.5	7.5	7.5
Isonicotinamide	OD	.672, .740	.461, .604	.474, .690
	µg	6.5	5.0	5.0
Nicotinamide	OD	.595, .590	.602, .579	.508, .435
	µg	5.5	5.5	4.5
Pyrazinamide	OD	.592, .575	.507, .520	.550, .586
	µg	5.0	5.0	5.0
Salicylamide	OD	.173, .113	.132, .084	.000, .120
	µg	1.0	1.0	0.5
Allantoin	OD	.165, .000	.000, .000	.096, .040
	µg	1.0	0.0	0.5
Succinamide	OD	.772, .947	.750, .830	.787, .826
	µg	7.5	6.5	7.0
Malonamide	OD	.217, .111	.092, .012	.000, .011
	µg	1.5	0.5	0.0

Legend same as in Table 4.

TABLE 4b

Amidase Activity of Representative Strains
Spectrophotometrically Determined

Substrate		SN14	SN14(B1)1	SN14(B1)I
Acetamide	OD	.627, .392	.524, .430	.617, .405
	µg	4.5	4.5	5.0
Benzamide	OD	.492, .484	.474, .480	.391, .452
	µg	4.5	4.5	4.0
Urea	OD	.883, .810	.896, .816	.931, .702
	µg	8.0	8.0	8.0
Isonicotinamide	OD	.492, .495	.297, 1484	.534, .450
	µg	4.5	4.0	4.5
Nicotinamide	OD	.772, .537	.474, .660	.623, .660
	µg	6.0	5.0	6.0
Pyrazinamide	OD	.552, .540	.597, .670	.455, .641
	µg	5.0	5.5	5.0
Salicylamide	OD	.455, .336	.000, .000	.074, .040
	µg	4.0	0.0	0.5
Allantoin	OD	.091, .000	.000, .000	.009, .000
	µg	0.0	0.0	0.0
Succinamide	OD	.892, .786	.786, .678	.880, .842
	µg	7.5	7.0	8.0
Malonamide	OD	.097, .128	.000, .000	.000, .000
	µg	1.0	0.0	0.0

Legend same as in Table 4.

TABLE 5

Summary of All Amidase Tests by

Comparison to Standards

Substrate	SN2	SN2(B1)1	SN2(B1)2	SN2(B1)3	SN2(B1)4
Acetamide	++	++	++	++	+
Benzamide	++	+	+	(+)	+
Urea	++	++	++	++	++
Isonicotinamide	++	+	++	+	(+)
Nicotinamide	++	++	++	+	++
Pyrazinamide	++	++	++	++	++
Salicylamide	+	0	0	0	0
Allantoin	0	0	0	0	0
Succinamide	++	++	++	++	++
Malonamide	(+)	0	0	0	0

Results are expressed according to the evaluation system of Bönicke (7,8) and Juhlin (25):

0 - <2 μg NH_3/ml = 0
2 - <3 μg NH_3/ml = (+)
3 - <5 μg NH_3/ml = +
5 -<10 μg NH_3/ml = ++
> 10 μg NH_3/ml = +++

Mean values of results are represented.

Standards prepared were: 2.0 μg NH_3/ml, 4.0 μg NH_3/ml, 6.0 μg NH_3/ml, and 8.0 μg NH_3/ml.

TABLE 5a

Summary of All Amidase Tests by Comparison to Standards

Substrate	SN10	SN10(B1)1	SN10(B1)2	SN10(B1)I	SN10(B1)II
Acetamide	+	++	+	+	++
Benzamide	++	+	+	+	+
Urea	++	++	++	++	++
Isonicotinamide	++	++	+	++	++
Nicotinamide	++	++	+	++	++
Pyrazinamide	++	++	++	++	++
Salicylamide	0	0	0	0	0
Allantoin	0	0	0	0	0
Succinamide	++	++	++	++	++
Malonamide	0	0	0	0	0

Legend same as in Table 5.

TABLE 5b

Summary of All Amidase Tests by Comparison to Standards

Substrate	SN14	SN14(B1)1	SN14(B1)2	SN14(B1)3	SN14(B1)I
Acetamide	++	++	(+)	+	+
Benzamide	++	+	(+)	+	+
Urea	++	++	++	++	++
Isonicotinamide	++	+	(+)	+	+
Nicotinamide	++	++	+	+	++
Pyrazinamide	++	++	+	++	++
Salicylamide	+	0	0	0	0
Allantoin	0	0	0	0	0
Succinamide	++	++	++	++	++
Malonamide	0	0	0	0	0

Legend same as in Table 5.

TABLE 6

Summary of Amidase Tests Read Spectrophotometrically

Substrate	SN2	SN2(Bl)3	SN2(Bl)4
Acetamide	++	+	++
Benzamide	+	+	+
Urea	++	++	++
Isonicotinamide	++	+	+
Nicotinamide	++	++	++
Pyrazinamide	++	++	+
Salicylamide	+	0	0
Allantoin	0	0	0
Succinamide	++	++	++
Malonamide	0	0	0

Results are expressed according to the evaluation system of Bönicke (7,8) and Juhlin (25).

0 - <2 μg NH_3/ml = 0
2 - <3 μg NH_3/ml = (+)
3 - <5 μg NH_3/ml = +
5 -<10 μg NH_3/ml = ++
>10 μg NH_3/ml = +++

Mean values (Table 4) are represented.

TABLE 6a

Summary of Amidase Tests Read Spectrophotometrically

Substrate	SN10	SN10(B1)1	SN10(B1)I
Acetamide	++	+	+
Benzamide	+	+	+
Urea	++	++	++
Isonicotinamide	++	++	++
Nicotinamide	++	++	++
Pyrazinamide	++	++	++
Salicylamide	0	0	0
Allantoin	0	0	0
Succinamide	++	++	++
Malonamide	0	0	0

Legend same as in Table 6.

TABLE 6b

Summary of Amidase Tests Read Spectrophotometrically

Substrate	SN14	SN14(B1)1	SN14(B1)I
Acetamide	+	+	++
Benzamide	+	+	+
Urea	++	++	++
Isonicotinamide	+	+	+
Nicotinamide	++	+	++
Pyrazinamide	++	++	++
Salicylamide	+	0	0
Allantoin	0	0	0
Succinamide	++	++	++
Malonamide	0	0	0

Legend same as in Table 6.

TABLE 7

Nitrate Reductase Activity
(by Comparison to Standards)

Strains	Test Number 1	2	3	Ave.
SN2	4	5	3	4
SN2 (B1) 1	1	1	1	1
SN2 (B1) 2	0	0	0	0
SN2 (B1) 3	0	0	0	0
SN2 (B1) 4	0	0	0	0
SN10	10	>10	> 5	10
SN10 (B1) 1	50	50	>50	50
SN10 (B1) 2	5	5	5	5
SN10 (B1) I	1	1	5	2
SN10 (B1) II	10	10	>10	10
SN14	10	10	10	10
SN14 (B1) 1	1	0	0	0
SN14 (B1) 2	0	0	0	0
SN14 (B1) 3	1	5	1	2
SN14 (B1) I	0	0	0	0

Results are expressed in μg $(NO_2)^-$ produced per ml of suspension after incubation for 2 hr at 37 C.

The average value is the mean value rounded off to the nearest μg.

Standards prepared were: 0.1 μg $(NO_2)^-$/ml, 1.0 μg $(NO_2)^-$/ml, 5.0 μg $(NO_2)^-$/ml, 10.0 μg $(NO_2)^-$/ml, and 50 μg $(NO_2)^-$/ml.

TABLE 8

Nitrate Reductase Activity
(Colorimetric Determination)

Strains	Test Number 1 mμmoles/ml	1 μg/ml	2 mμmoles/ml	2 μg/ml	Ave. (to nearest .5 μg) mμmoles/ml	Ave. (to nearest .5 μg) μg/ml
SN2	16.8	3.4	18.0	3.5	17.4	3.5
SN2 (B1) 3	0.0	0.0	0.0	0.0	0.0	0.0
SN2 (B1) 4	0.0	0.0	0.0	0.0	0.0	0.0
SN10	55.0	10.1	52.0	10.0	53.5	10.0
SN10 (B1) 1	>100.0	>20.0	>100.0	>20.0	>100.0	>20.0
SN10 (B1) I	2.8	0.5	1.4	0.3	2.1	0.4
SN14	47.7	9.6	38.6	7.8	44.6	8.8
SN14 (B1)	0.0	0.0	0.0		0.0	0.0
SN14 (B1) I	0.0	0.0	0.0		0.0	0.0

To convert mμmoles $(NO_2)^-$ produced/ml to μg $(NO_2)^-$ produced/ml, Beer's law was used: $O.D. = k_1 A_{Klett} = \varepsilon CL$

$$C = \frac{k_1 A_{Klett}}{\varepsilon L}$$

$$\frac{k_1}{\varepsilon L} = .021 \text{ (from Fig. 2)}$$

$$C = .021\, A_{Klett}$$

Where $C = \mu g\ (NO_2)^-/ml$

A_{Klett} = Klett units = 10 mμmoles/ml

TABLE 9

Phenolphthalein Sulfatase Activity

Strain	Time of Incubation at 37 C					
	3 day	21 day	14 day			14 day (Ave.)
			#1	#2	#3	
SN2	0	20	>6	8	8	8
SN2(B1)1	0	16	6	6	6	6
SN2(B1)2	0	16	0	0	0	0
SN2(B1)3	0	20	1	1	0	1
SN2(B1)4	0	20	1	0	1	1
SN10	0	16	<6	<6	>2	5
SN10(B1)1	0	16	2	1	2	2
SN10(B1)2	0	16	<6	<6	<6	5
SN10(B1)I	0	20	>16	20	20	20
SN10(B1)II	0	16	0	0	0	0
SN14	0	20	16	8	16	12
SN14(B1)1	0	16	0	0	0	0
SN14(B1)2	0	20	6	>6	6	6
SN14(B1)3	0	16	0	0	0	0
SN14(B1)I	0	20	6	2	<6	4

Results are expressed in µg phenolphthalein produced per ml of solid medium.

The average value is the mean rounded off to the nearest µg.

Standards used were: 0.0 µg, 1.0 µg, 2.0 µg, 6.0 µg, 16.0 µg, and 40.0 µg phenolphthalein per ml.

TABLE 10

Malachite Green Reductase Activity

Strains	Time Required for Growth (Days)		Decolorization Ability	
	Solid	Liquid	Solid	Liquid
SN2	3	7	-	+
SN2(B1)1	3-4	10	+	+
SN2(B1)2	3-4	10	+	+
SN2(B1)3	5-6	10	+	+
SN2(B1)4	5-6	10	+	+
SN10	3	7	-	+
SN10(B1)I	3-4	7	-	+
SN10(B1)2	5-6	10	+	+
SN10(B1)I	5-6	10	+	+
SN10(B1)II	5-6	10	+	+
SN14	3	7	-	+
SN14(B1)1	5-6	10	+	+
SN14(B1)2	5-6	10	+	+
SN14(B1)3	5-6	10	+	+
SN14(B1)I	5-6	10	+	+

+ Indicates ability to convert malachite green to its leuco form (9) as indicated by the color of the medium after noticeable growth had occurred.

The tests were performed in duplicate. The results of the duplicate tests were identical.

DISCUSSION

The amidase spectra of parent strains herein reported were qualitatively in good agreement with previously published data (8). *Mycobacterium smegmatis* was earlier reported to have variable salicylamidase activity (8,23). The unstability of the character invalidates any proposition that decrease of salicylamidase activity of strains *M. smegmatis* SN2 and SN14 due to B1 lysogeny was of taxonomical significance.

It was difficult to compare results concerning the nitrate reductase activity of parental strains with previous reports, since either a different method was employed or different species were tested. Neither Virtanen (38) nor Kubica and Dye (25) related their results to standards of specific concentration, but rather compared enzymatic activity in different species to arbitrary standards, "photometer drum readings" and color standards, respectively. Juhlin (23) used a four hr incubation period but found variable activity in *M. smegmatis* similar to the present work. Jones and White (20) used *M. smegmatis* ATCC 607, referred to in the present work as SN10, and found a reductase activity of over 30 mμmoles nitrate/ml. This roughly corresponds to 10 μg nitrite/ml, the value obtained for SN10 and SN14 in this work (Tables 7, 8). They also found that lysogenization of *M. smegmatis* ATCC 607 with mycobacteriophage D_{29} increased nitrate reduction only in 50% of the strains while lysogenization of the same strains with mycobacteriophage B4 increased the nitrate reductase activity of nearly

all strains. In the course of this study a similar increase was found only in one B1-lysogenic strain of SN10. On the contrary, decreased activity as reported by Mankiewicz (28), was the rule following lysogenization of all the other strains.

Even greater difficulty was encountered in trying to compare phenolphthalein sulfatase activity of parent strains with previously published data on *M. smegmatis*. Sommers (34) stated that *M. smegmatis* gave a positive "arylsulfatase" test in 14 days. He did not elaborate further. Whitehead *et al.* (41) compared rapidly growing mycobacterial species but did not report the amount of phenolphthalein produced. Juhlin (23) used an altogether different method than this author for his test. Jones and White (20), as well as Wayne (39), used the three day test and both agreed that *M. smegmatis* was negative.

In both the amidase and nitrate reductase tests the use of a spectrophotometer or colorimeter to evaluate the concentration of reaction products, proved much superior to the rather subjective method of visual comparison of color reaction with standards of known concentration. In the former case bacteria had to be removed by centrifugation and the supernatant alone was tested. This is the probable explanation for the generally lower values of NH_3/ml reported by Juhlin (23) using a spectrophotometric method. The presence of bacteria tended to make the suspension "milky" in the test tubes, thus rendering visual comparison to standards especially subjective when low amounts of NH_3 (1-3 µg/ml) were produced.

Evaluation of the changes in enzymatic activity upon B1-lysogeny suggested that some correlation exists between nitrate reductase and phenolphthalein sulfatase activity on the one hand, and salicylamidase activity on the other hand. Lysogeny for phage B1 in strains SN2 and SN14, which originally possessed detectable salicylamidase activity, resulted in the decrease of both nitrate reductase, phenolphthalein sulfatase and salicylamidase activity. On the other hand, in SN10 which did not show any detectable salicylamidase activity, lysogenization with phage B1 had variable effects on these activities. This phenomenon could not be explained by transduction since the propagating host for phage B1 was *M. smegmatis* SN2 and the above mentioned changes were observed not only in strain SN14 but in SN2 as well.

One may speculate that if the locus determining salicylamidase activity served as the integration site for phage B1 on the bacterial chromosome, its absence may have caused the phage to integrate in a different manner or at different sites. Unfortunately, however, nothing is known about the chromosome map of either mycobacteria or mycobacteriophages, or the exact mechanism of integration of the mycobacteriophage genome into the host chromosome.

The results of the malachite green reduction tests showed that the egg constituent of the Loewenstein-Jensen medium affected this activity (Table 10). They also showed that, while no mechanism for the reaction

can be presently proposed, the decolorization of the Loewenstein-Jensen medium by *M. smegmatis* lysogenized with B1 involved more than just a reversible reduction of malachite green which this terminology (9) seemed to imply.

Altered colonial morphology as a result of B1 lysogeny in *M. smegmatis* was reported for *M. smegmatis* ATCC 607 lysogenized with D29 or B4, by Jones and White (20). Similar alteration of morphology could be observed even in non-lysogenic strains (20). Several strains tested in the present work which did not produce phage but were resistant to phage also possessed a similar altered morphology. Consequently altered morphology may have been a manifestation of phage resistance, without lysogeny. However in the course of the present work lysogens were observed with colonial morphology characteristic of the parental strain, and in Bönicke's studies, loss of prophage did not effect reversion to the original morphology (9). Therefore neither resistance to phage nor lysogeny seemed to be strictly related to altered colonial morphology. It appeared to be a reasonable conclusion therefore that the relationship between altered colonial morphology and lysogeny at present is unclear.

Lysogenization of *M. smegmatis* strains SN2, SN10, and SN14 thus has been shown to affect to a considerable degree the enzymatic activity of these strains. In addition to decreased salicylamidase, nitrate reductase and phenolphthalein sulfatase activity, and positive malachite green

reduction reported by this author, decrease in the catalase and acetamidase activity as well as in the ability to "transform" ammonium ferrocitrate, and to utilize paraffin as a sole carbon source were attributed to lysogeny by Mankiewicz and others (28). If various phages can produce such radical changes in their mycobacterial hosts under laboratory conditions, it is not unreasonable to assume that they play an important role in the natural variability of *Mycobacterium* species, possibly a greater one than in any other genera.

The significance of this research is that it can explain the difficulty encountered in classifying certain mycobacterial strains which are isolated from time to time in diagnostic laboratories. Most often these isolates show resistance to the known mycobacteriophages and display enzymatic activities different from that of the known rapidly growing mycobacterial species. Although attempts to isolate phage from them usually fail, the non-availability of susceptible indicator strains or the defectiveness of the prophage they carry may account for this failure.

SUMMARY

Mycobacterium smegmatis strains SN2, SN10, and SN14 were lysogenized with mycobacteriophage smegmatis B1. Lysogeny was determined by two criteria: immunity to superinfection by phage B1, and production of plaques by the lysogen when plated against its phage-susceptible parent strain. Lysogens and their parents were then tested for acetamidase, benzamidase, urease, isonicotinamidase, nicotinamidase, pyrazinamidase, salicylamidase, allantoinase, succinamidase, and malonamidase activity using a 22 hr amidase test. They were also tested for phenolphthalein sulfatase activity upon incubation for 3, 14, and 21 days, for nitrate reductase activity using a 2 hr test, and for the reduction of malachite green in Loewenstein-Jensen medium. Lysogeny in strains SN2 and SN14, which originally possessed appreciable salicylamidase activity, resulted in decreased salicylamidase, phenolphthalein sulfatase, and nitrate reductase activity as well as in the ability to decolorize malachite green in Loewenstein-Jensen medium. However in lysogens derived from strain SN10, which showed no appreciable salicylamidase activity, changes varied in nitrate reductase and phenolphthalein sulfatase activity and one strain did not decolorize malachite green at all. Since no chromosome map has been constructed for mycobacteria or their phages and the exact mechanism of mycobacteriophage integration is not known, it can only be hypothesized that phage B1 integrates at or near the salicylamidase locus. Absence of a salicylamidase locus thus may cause

the phage to integrate in a different manner or at alternate sites. Transduction could not have accounted for the observed variations since the reported changes have been observed not only in strain SN14 but in SN2 as well and SN2 served as the host for lytic propagation of phage B1 ever since its isolation. Altered colonial morphology was observed in some lysogenic strains but could not be directly related to lysogeny.

ACKNOWLEDGMENTS

I would like to thank Dr. S. E. Juhasz for his guidance and assistance throughout this work. I would also like to express my gratitude to Dr. Monique Harize who helped prepare the lysogens and whose assistance and encouragement during the long hot summer months gave me the optimism necessary to complete the work, and to Dr. R. Bönicke whose advice through correspondence, and reagents helped make the methods reproducible.

Last but not least, my thanks to Roberta Novak who typed this thesis for me, and to Deanne Jungels who typed the formal outline.

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APPROVAL SHEET

The thesis submitted by Sheldon Gelbart has been read and approved by the director of the thesis.

Furthermore, the final copies have been examined by the director and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the thesis is now given final approval.

The thesis is therefore accepted in partial fulfillment of the requirements for the degree of Master of Science.

June 13, 1969
Date

Lloyd E. Jukes
Signature of Advisor