A MUTANT OF A SCOTOCHROMOGENIC *MYCOBACTERIUM* DETECTED BY COLONY MORPHOLOGY AND LIPID STUDIES

G. B. FREGNAN[1], D. W. SMITH, AND H. M. RANDALL

Department of Medical Microbiology, University of Wisconsin, Madison, Wisconsin, and Randall Laboratory of Physics, The University of Michigan, Ann Arbor, Michigan

Received for publication October 31, 1961

ABSTRACT

FREGNAN, G. B. (University of Wisconsin, Madison), D. W. SMITH, AND H. M. RANDALL. A mutant of a scotochromogenic *Mycobacterium* detected by colony morphology and lipid studies. J. Bacteriol. **83**:828–836. 1962.—A variant of a scotochromogenic strain of *Mycobacterium* is described. This mutant was recognized by studies of colony morphology and lipid content. Mycoside D was shown to be present in the smooth-Sy variety but absent in the rough-Ry variety. The rough-Ry variety appeared spontaneously from smooth-Sy, or by induction after prolonged incubation in a synthetic liquid medium or exposure to ultraviolet irradiation. This mutation is not reversible.

In previous publications (Fregnan, Smith, and Randall, 1961*a*,*b*; Smith et al., 1961), a close relationship was described between colony morphology and mycoside content in *Mycobacterium kanasii*, *M. fortuitum*, and scotochromogenic mycobacteria. Attention was called (Fregnan et al., 1961*a*) to the observation that one scotochromogenic strain contained mycoside D (a specific glycolipid-peptide) in 1956, but later attempts (1957–1959) to demonstrate this compound in the same strain were unsuccessful. One of the possible explanations of this fact was that the loss of the specific mycoside could be due to a mutant form, lacking this compound, which became the dominant form in the culture. This hypothesis has been shown to be correct.

MATERIALS AND METHODS

Strain. The strain (P-5) was obtained from E. H. Runyon, first in 1956 and later in 1959.

Colony morphology and mass culture. Techniques for the study of colony morphology and for mass culture were described previously (Fregnan et al., 1961*b*). The stability and homogeneity of a given colony form were confirmed by at least three successive passages of a single colony inoculum on oleic acid-albumin agar medium (OAA agar) containing cycloheximide.

Colony type symbols. This terminology was introduced in a previous paper (Fregnan et al., 1961*b*) and extended and modified slightly in a later one (Fregnan and Smith, 1962).

Animal inoculation. Cultures were grown in Tween-albumin broth at 37 C for 7 days and were diluted to 10^{-2}. Guinea pigs were inoculated subcutaneously over the sternum with 1.0 ml of this suspension; 4-week-old chickens (Barred Rock) were given 0.4 ml intravenously; mice (Swiss-Webster strain) were inoculated intraperitoneally with 0.4 ml. The animals were killed 2 months after infection, and the tissues examined for evidence of gross tubercular lesions.

Ultraviolet irradiation. Mycobacteria seeded on OAA agar were exposed to unfiltered ultraviolet light (Mineralight SL 3660, Ultraviolet Products, Inc., South Pasadena, Calif.) for 10 to 20 min every 12 hr for the first few days of incubation.

Extraction of lipids. Mycobacteria, grown for 15 to 30 days at 37 C on synthetic liquid media, were harvested either directly or after killing by exposure to 2% phenol for 48 hr at 37 C. The separation of the cells from the medium was accomplished by aspiration from pellicle cultures or by filtration or centrifugation of dispersed cultures. The moist cells were extracted three times with ethanol-ether (50:50, v/v) by continuous agitation at 25 C. The duration of the first extraction for nonphenolized cultures was 48 hr; otherwise, the extraction periods were 3 hr.

The pooled extracts, filtered through coarse sintered glass, were dried under vacuum at 37 C. The dried lipids were redissolved in chloroform. A salt solution, recommended by Folch, Lees, and Sloan Stanley (1957) for obtaining maximal

[1] Present address: Istituto di Microbiologia, Universitá di Bologna, Bologna, Italy.

recovery of lipids, was added, and the two layers were separated in a separatory funnel at either 25 or 5 C. The chloroform layer was dehydrated by leaving it in contact with $MgSO_4$ (anhydrous) for 24 to 48 hr; the chloroform was removed under vacuum at 37 C. By these procedures, 4 to 8% of the lipids were extracted from this culture. Before chromatography, the ethanol-ether extracted lipids were further separated into soluble and insoluble fractions by extraction with boiling acetone. The hot acetone-insoluble fractions, containing the phosphatides, were not chromatographed.

Chromatography. Columns, 2.5 cm in diameter, were packed with a slurry of 60 g of Florisil in hexane-benzene (50:50, v/v) with the aid of air pressure (5 psi). The hot acetone-soluble lipids, 1 to 2 g dissolved in a few ml of hexane-benzene (50:50, v/v), were added to the packed column, and the elution was begun with the same solvent and continued according to the sequence given by Smith et al. (1960). Then 100 ml of each solvent were collected; the elution with any given solvent was continued until the amount of material eluted in a fraction was less than 1 mg.

The solvents were removed and infrared spectra were recorded for each fraction, according to the procedure described earlier (Smith et al., 1960). Classification of the eluted components was made by comparison of their infrared spectra with those of substances previously recognized in mycobacteria and with those of known compounds.

RESULTS

First study of culture P-5 in 1956 with the recognition of mycoside D. The procedure followed in the first study of strain P-5 was described by Smith et al. (1957). The inoculum taken from American Trudeau Society (ATS) medium was seeded on 100 ml of malic acid medium and incubated stationary at 37 C. The first appearance of a thin surface growth was noted only after 2 months incubation, when numerous cells were dispersed throughout the medium. For mass culture, an inoculum taken from the thin surface pellicle was seeded in 20 flasks, each containing 700 ml of the same medium. This culture was harvested after 7 weeks at 37 C, and was composed of a thin surface growth and a mucoid, golden-yellow bottom growth. Mycoside D was demonstrated in the lipids from these cells, and was eluted from the column by ether-methanol (80:20, v/v). Reference to the spectra of this column now suggests the presence of another substance, tentatively called component X, present in small amounts in several fractions eluted in ether-methanol (95:5, v/v). No studies of colony morphology of this strain were done in 1956.

Subsequent studies of strain P-5 (1957–1959) and the disappearance of mycoside D. During this period of our studies, an inoculum on malic acid medium was stored at 5 C for cultures that might later be required for mass culture. When a sub-

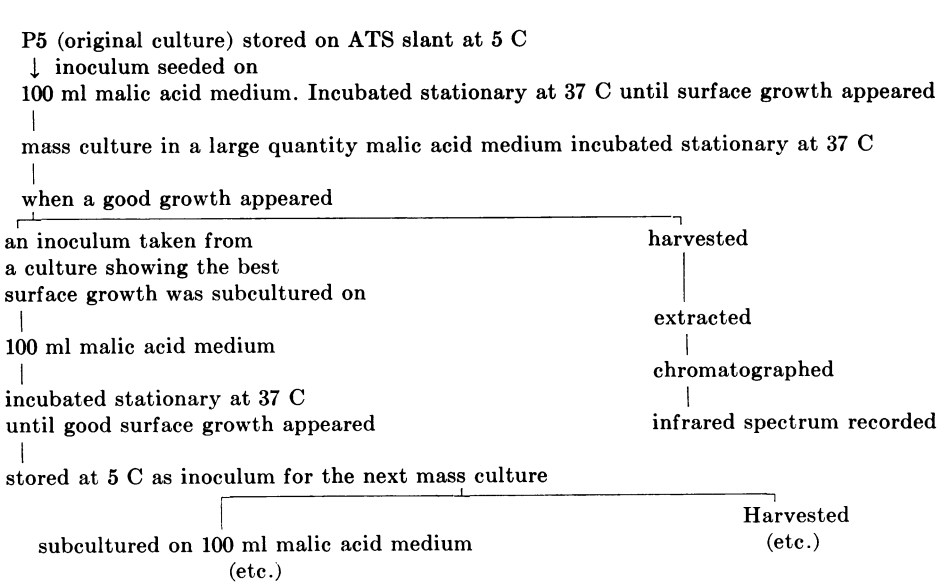

FIG. 1. *Scheme of the mass culture and extraction procedure followed in 1956–1958*

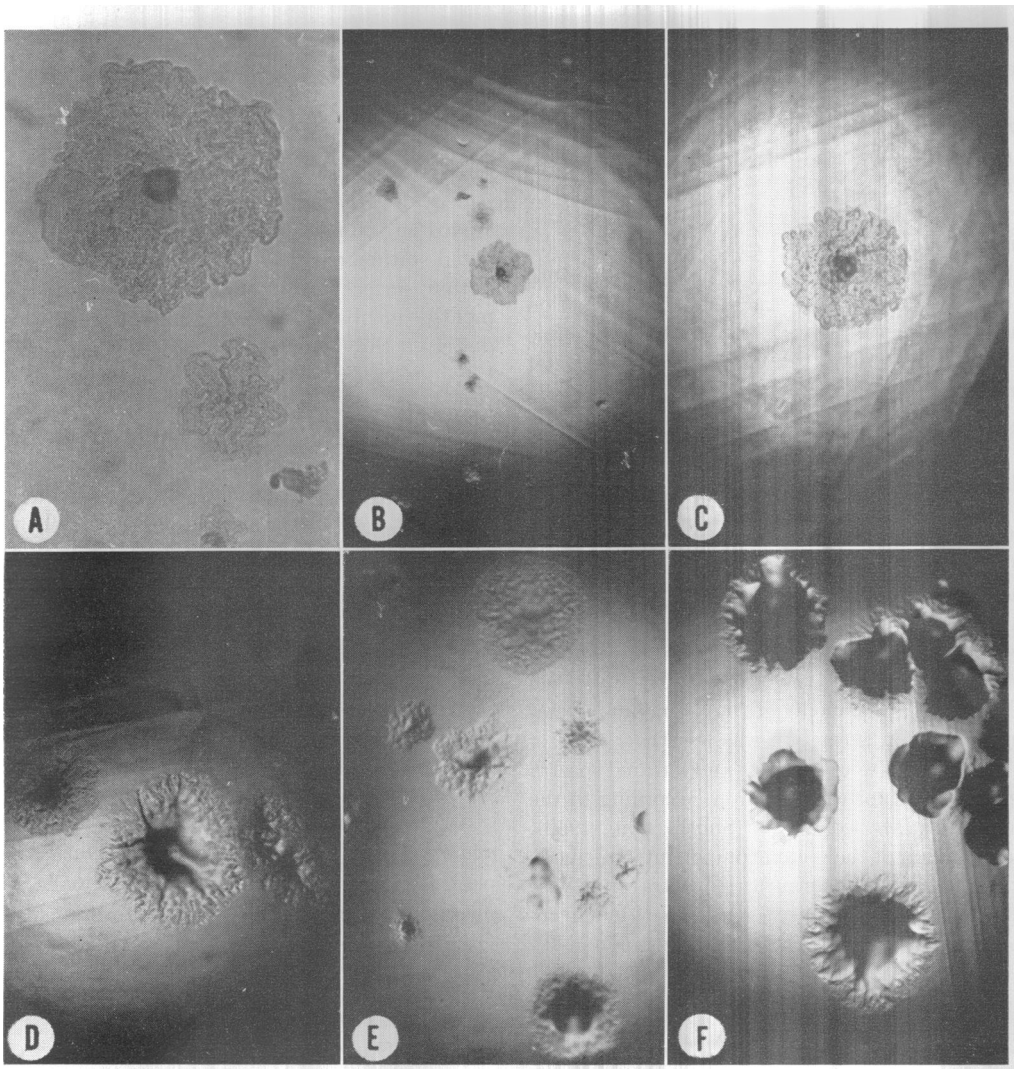

FIG. 2. *Photomicrographs of the stepwise development of smooth-Sy.* A, *rough-Cs (240 ×) 6–12 days;* B, *rough-Cs (24 ×) 6–12 days;* C, *transition stage rough Cs–smooth Sy (24 ×) 12–15 days;* D, *smooth-Sy wrinkled (24 ×) 15–18 days;* E, *smooth-Sy wrinkled (24 ×) 15–18 days;* F, *smooth-Sy (24 ×) 21–30 days.*

sequent mass culture was seeded from this inoculum, and the surface growth had developed upon incubation, one flask with a good surface growth was again separated and stored at 5 C. From 1957 to 1959, seven separate mass cultures of strain P-5 were prepared, each in turn inoculated from the previous growth stored for an interval at 5 C (Fig. 1). No mycoside D was demonstrated in the lipids extracted from any of these cultures. It was noted that the character of the growth changed from a moist surface-subsurface growth in the beginning to a progressively drier pellicle type of growth. This suggested the possibility that a change had occurred in the bacterial population during the numerous passages on this medium. On the basis of these observations, we undertook a study of the colony morphology and mycoside content of the original culture maintained on ATS medium.

Colony morphology studies 1960–1961. An inoculum taken from the ATS stock culture of strain P-5 was dispersed in Tween-albumin medium, and dilutions of this suspension were plated on OAA agar. Study of the colony morphol-

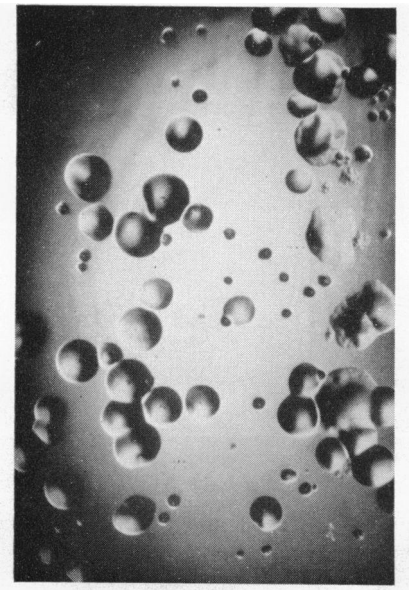

FIG. 3. *Photomicrograph of smooth-Dy colonies which result from overcrowding.*

ogy revealed a heterogeneous population made up of two distinct colony forms. These two types, smooth-Sy and rough-Ry (Fig. 2F and 4F), were isolated in pure culture and were shown to be stable and reproducible on solid medium.

Figure 2 presents photomicrographs of the stepwise development of a smooth-Sy colony on OAA agar, from the first appearance of growth to maturity. The following observations were made on the morphology of the colonies with the plates in the inverted position. In 3 to 6 days, microcolonies are observed. After 6 to 12 days, the colonies have the general features of rough-Cs, but the microscopic color is somewhat lighter and the cords perhaps less evident. After 12 to 15 days, a transition stage from rough-Cs to smooth-Sy occurs. The filaments are growing so close together that they lose their individuality. The dark central spot is still visible. The microscopic color ranges from light gray to yellowish; macroscopically, it appears cream-colored. After 15 to 18 days, the colonies could already be considered smooth-Sy, but they are still flat and very wrinkled. The microscopic and macroscopic color is pale yellow. By 18 to 21 days, the colonies are getting smoother and assuming the features characteristic for smooth-Sy. The microscopic and macroscopic color is yellow. Within 21 to 30 days, smooth-Sy is evident.

It has been observed that if the inoculum on OAA agar was heavy, the crowded smooth-Sy colonies had a different form, which we have called smooth-Dy (Fig. 3). This change was phenotypic because, once reseeded on OAA agar at greater dilution, they regain their characteristic smooth-Sy morphology.

The photographs in Fig. 4 illustrate the step by step growth of a rough-Ry type of colony that is described as follows: 3 to 6 days, microcolonies; 6 to 9 days, rough-Cs; 9 to 12 days, still rough-Cs but the microscopic color is changing to yellowish gray and macroscopically it appears cream-colored. In 12 to 18 days, some colonies are developing into rough-Ry. The central spot and the cords are becoming less distinct; thus, the structure changes from a serpentine type into a complex arborescent one with filaments developing in all directions. The microscopic color is yellow-gray; macroscopically, it is pale yellow. In 18 to 24 days, some colonies have completed the transition to rough-Ry. The microscopic color is yellow-gray; the macroscopic color is yellow. In 24 to 30 days, rough-Ry and a few younger colonies still maturing are evident.

Animal pathogenicity studies of smooth-Sy and rough-Ry. Guinea pigs, chickens, and mice, inoculated 8 weeks earlier with 7-day cultures of smooth-Sy and rough-Ry, were killed; study of the tissues of these animals revealed no evidence of gross tuberculosis.

Chemical studies of the lipids of smooth-Sy and rough-Ry types. Inocula taken from typical smooth-Sy and rough-Ry colonies were grown in mass culture on sodium glutamate-albumin medium for 3 or 7 weeks, following the technique described earlier for *M. kansasii* (Fregnan et al., 1961*b*). It was possible to extract twice as much total lipid from rough-Ry as from smooth-Sy, but the hot acetone soluble and insoluble fractions of the two samples contained approximately the same percentage of lipids (Table 1). Table 2 and Fig. 5 summarize the results obtained after chromatography on Florisil of the hot acetone-soluble fractions of the lipids derived from the two colony types. The lipids from smooth-Sy were shown to contain mycoside D (previously called JAT, Smith et al., 1960), a glycolipid-peptide specific for some scotochromogenic mycobacteria. Mycoside D has an infrared spectrum similar to but distinct from mycoside C (glycolipid-peptide formerly called JAV, found in some

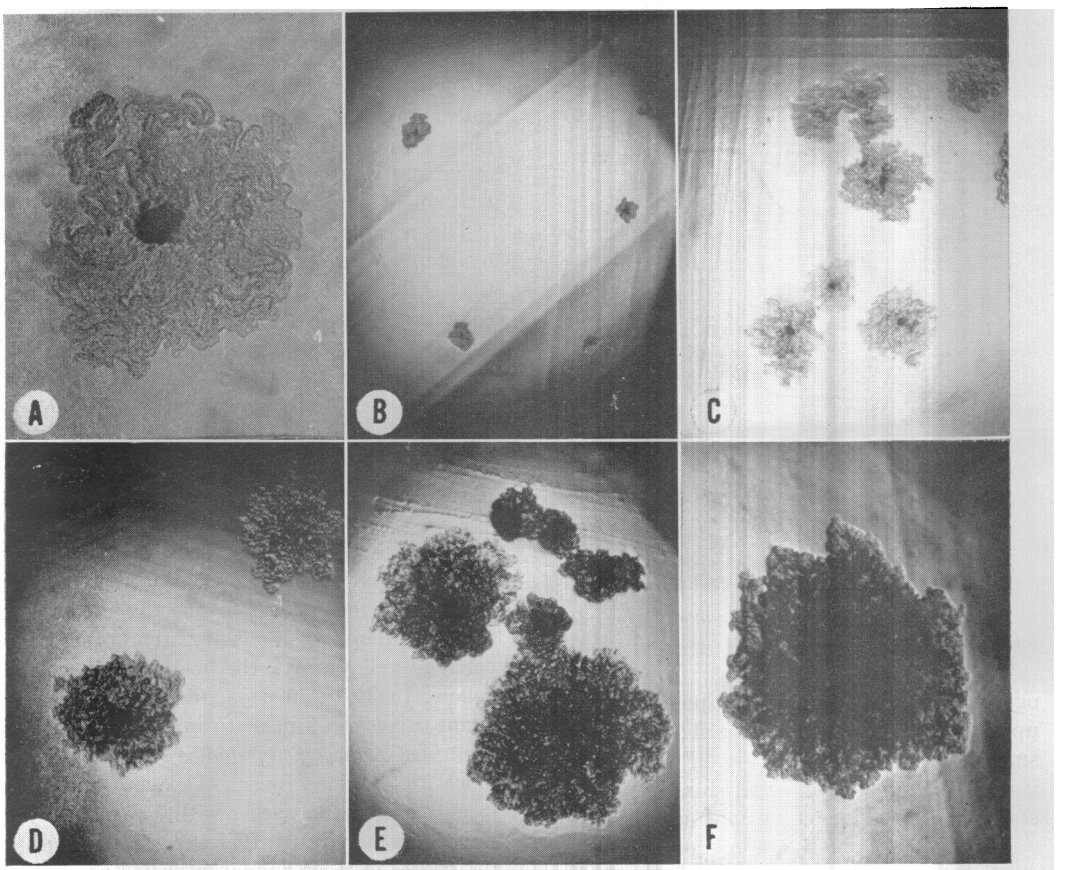

FIG. 4. *Photomicrographs of the stepwise development of rough-Ry.* A, *rough-Cs (240 ×) 6–9 days;* B, *rough-Cs (24 ×) 6–9 days;* C, *rough-Cs (24 ×) 9–12 days;* D, *transition of rough-Cs to rough-Ry (24 ×) 12–18 days;* E, *transition of rough-Cs to rough-Ry (24 ×) 18-24 days*; F, *rough-Ry (24 ×) 24–30 days.*

avian strains) and mycoside Cm, detected by Chaput, Michel, and Lederer (1961) in the lipids of *M. marianum.* No spectroscopic evidence was seen for the presence of component X in any of the fractions eluted from the column charged with the lipids of the smooth-Sy colony form, whereas the lipids extracted from the culture of colony type rough-Ry did show the presence of infrared absorption bands characteristic for component X in the fractions eluted in ether-methanol (95:5, v/v). No mycoside D was eluted from the rough-Ry lipids. All the other substances listed in Table 2 were shared equally by both types.

Figure 6 compares the elution curves for columns charged with smooth-Sy lipids and rough-Ry lipids. The most obvious difference is in the ether-methanol (80:20, v/v) fractions, and is accounted for by the difference in the occurrence of mycoside D.

TABLE 1. *Percentages of lipids extracted from smooth-Sy and rough-Ry colonies**

Colony type	Total lipids	Acetone-soluble	Acetone-insoluble
	%	%	%
Smooth-Sy	4–3	85–90	10–15
Rough-Ry	8–7	85–90	10–15

*Extraction was with ether-methanol (50:50 v/v). Total lipids are expressed as g/g extracted dry cells. The fractions soluble and insoluble in hot acetone are expressed as percentages of the total lipids.

Induced dissociation of smooth-Sy to rough-Ry. Two possible explanations for the occurrence of rough-Ry in our stock culture of strain P-5 were that it was introduced as a contaminant and slowly became predominant by serial passage on malic acid medium, or that it is a mutant which

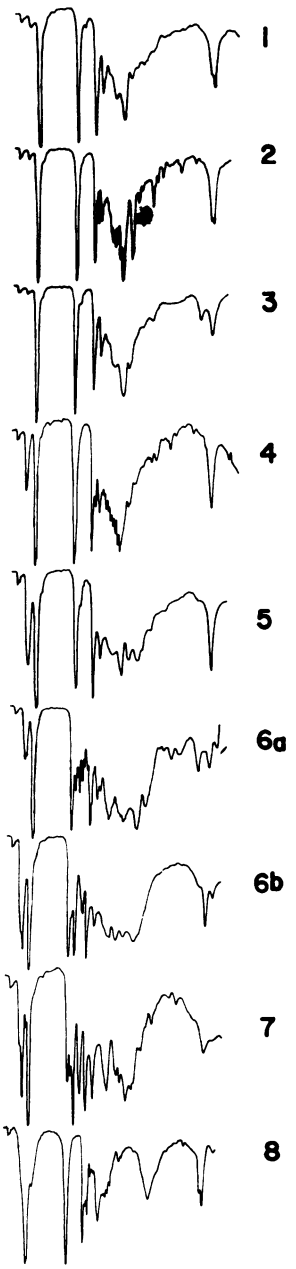

FIG. 5. *Infrared spectra (from 2 to 15 μ) of components listed in Table 2.*

spontaneously arises. To study these possibilities further, two types of experiments were performed with the pure cultures of smooth-Sy and of rough-Ry: passage in malic acid medium with prolonged incubation, and exposure to ultraviolet irradiation for different periods of time.

Neither of these two procedures caused any change in the characteristic colony form rough-Ry. However, the smooth-Sy colony, shown to be stable by three passages of a single colony on OAA agar, gave rise to colonies of the rough-Ry variety either after prolonged incubation in malic acid medium or after exposure to ultraviolet light. Table 3 gives the details of the type of experiment conducted in malic acid medium.

The change from smooth-Sy to rough-Ry is also associated with an increase in the relative amount of lipid present in the mycobacteria (Table 1) and a consequent change in the aspect of the culture, from hydrophilic to hydrophobic, so that the rough-Ry variety gives a better surface growth on malic acid medium.

DISCUSSION

Investigations conducted thus far have failed to demonstrate that mutations from one species to another occur among *M. tuberculosis*, *M. bovis*, and *M. avium*; however, mutations within species have been successfully demonstrated.

In this paper is described a variant in a scotochromogenic strain of a mycobacterium which arose spontaneously or by induction. A rough-Ry type of colony was obtained from a smooth-Sy type. The mutation was associated with the loss of mycoside D and the acquisition of another component, called X, not yet isolated in pure form. We were unable to demonstrate the reversibility of this mutation.

A close relationship is seen between this dissociation and the classical S → R type described for other bacteria by Arkwright (1920). However, at the present time we do not have sufficient basis to use these terms. Wilson and Miles (1955) gave the following criteria that define the R variant as different from the S: (i) loss of the antigenic component characterizing the surface of the bacterial cell in the normal smooth form, whether this component is normally present in the form of a bacterial capsule or not; (ii) loss of virulence, partial or complete; (iii) altered sensitivity to various bacteriophages; (iv) a change in colony form, usually, but not always, in the direction of increased granularity or roughness; (v) a change in the hydrophobic or hydrophilic properties of the cell, usually but not always in the direction of a decreased affinity for water, and a consequently increased sensitivity to the flocculating action of electrolytes; and (vi) in-

TABLE 2. *Infrared spectra of major components eluted from Florisil columns charged with lipids of smooth-Sy and rough-Ry varieties*

Solvent system	Smooth-Sy		Position of infrared spectrum (Fig. 5)	Rough-Ry	
	Amount	Nature of the main components		Nature of the main components	Amount
	%				%
Hexane-benzene (50:50, v/v)	7	Esters of fatty acids	1	Esters of fatty acids	8
Benzene	50	Triglyceride of fatty acids	2	Triglyceride of fatty acids	47
Benzene-ether (95:5, v/v)	14	Triglyceride of unsaturated fatty acids	3	Triglyceride of unsaturated fatty acids	15
Benzene-ether (50:50, v/v)	6	Diglyceride of stearic and palmitic acids	4	Diglyceride of stearic and palmitic acids	10
Ether-methanol (99:1, v/v)	2	Monoglycerides of mycolic and other acids	5	Monoglycerides of mycolic and other acids	2.5
Ether-methanol (99:5, v/v)	1.5		6a	Component X	2
		Amides	6b	Amides	
Ether-methanol (80:20, v/v)	7	Glycolipid-peptide (mycoside D)	7		1.5
Ether-acetic acid (99:1, v/v)	4	Fatty acids	8	Fatty acids	5
Lipids not eluted	8.5	Not studied	Not studied	Not studied	9

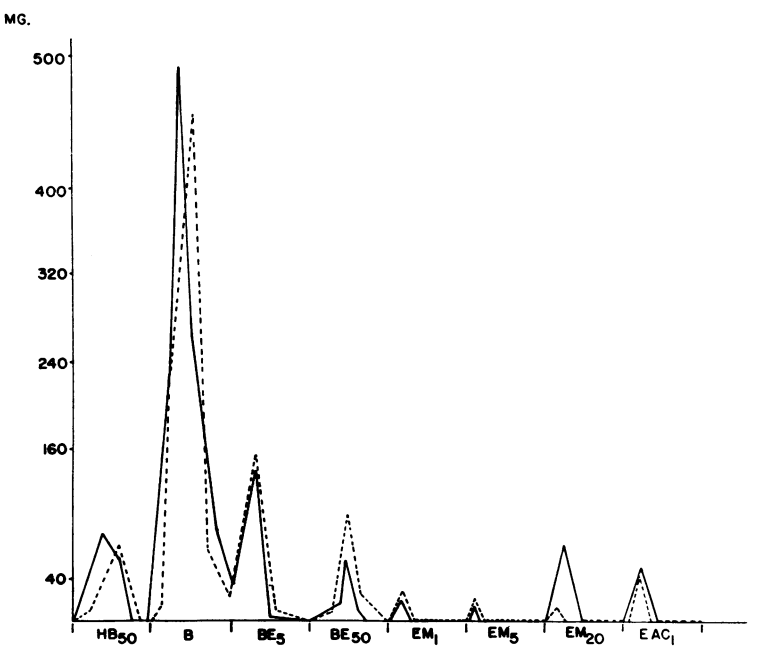

FIG. 6. *Chromatographic elution curves for the hot acetone-soluble lipids of smooth-Sy (continuous line) and rough-Ry (dashed line).*

creased susceptibility to the bactericidal action of normal serum.

According to this definition, we cannot now consider the dissociation, here described, as an S → R dissociation. Similarities are seen that would make us predict the final adoption of the terminology S → R to describe this mutation, but more work needs to be done. We demonstrated

TABLE 3. *Dissociation of smooth-Sy into rough-Ry in malic acid medium*

Period checkup of the cultures in 100 ml malic acid medium incubated stationary at 37 C after	Strains transferred into 100 ml malic acid medium, incubated stationary at 37 C								
	P-5 original culture, from ATS to			Smooth-Sy from OAA agar to			Rough-Ry from OAA agar to		
	Growth pattern	Colony form on OAA agar	Specific compound after mass culture in malic acid of an inoculum taken from the surface	Growth pattern	Colony form	Specific compound after mass culture in sodium glutamate	Growth pattern	Colony form	Specific compound after mass culture in sodium glutamate
month									
0	Dispersed yellow thin growth	$\frac{\text{smooth-Sy}}{\text{rough-Ry}} = \frac{1000^*}{1}$	Myco-side D	Dispersed yellow thin growth	smooth-Sy	Myco-side D	Thin surface growth	rough-Ry	Compo-nent X
1	Dispersed yellow mucoid growth	$\frac{\text{smooth-Sy}}{\text{rough-Ry}} = \frac{1000^*}{1\text{–}2}$	Myco-side D	Dispersed yellow mucoid growth	smooth-Sy	Myco-side D	Surface granular yellow pellicle	rough-Ry	Compo-nent X
2	Very thin yellow pellicle on the surface and yellow submerged mucoid growth	$\frac{\text{smooth-Sy}}{\text{rough-Ry}} = \frac{1000^*}{4\text{–}5}$	No specific compounds	Dispersed yellow mucoid growth	smooth-Sy	Myco-side D	Thick granular yellow-orange pellicle	rough-Ry	Compo-nent X
3	Thick granular yellow-orange pellicle and yellow submerged mucoid growth	$\frac{\text{smooth-Sy}}{\text{rough-Ry}} = \frac{100^*}{5}$	Not studied	Very thin pellicle on the surface and dispersed yellow mucoid growth	$\frac{\text{smooth Sy}}{\text{rough-Ry}} = \frac{1000^*}{1\text{–}2}$	Not studied	Thick granular yellow-orange pellicle and many cells deposited on the bottom	rough-Ry	Not studied
4	Thick granular yellow-orange pellicle and submerged growth, some lying on the bottom	$\frac{\text{smooth-Sy}}{\text{rough-Ry}} = \frac{100^*}{50}$	Not studied	Thin pellicle on the surface and dispersed yellow mucoid growth	$\frac{\text{smooth Sy}}{\text{rough-Ry}} = \frac{1000^*}{2\text{–}3}$	Not studied	Thick dry orange pellicle and many cells deposited on the bottom	rough-Ry	Not studied

* By approximation only.

that the smooth-Sy type dissociated into the rough-Ry type, acquiring an increased granularity and roughness and changing from hydrophilic to hydrophobic, with a consequent increased sensitivity to the flocculating action of electrolytes. We did not see any variation in virulence because neither variety is virulent for the animals studied. It will be of interest to determine any change in sensitivity to bacteriophages or to the bactericidal action of normal serum. It also remains to be seen if the disappearance of mycoside D and the appearance of component X in the lipids of the two varieties are related to the loss of an antigenic component characterizing the surface of the bacterial cell.

A new terminology to describe colony forms was used in this paper, for the reasons given in the accompanying publication (Fregnan and Smith, 1962).

ACKNOWLEDGMENT

This work was supported by grants E-646 and E-1948 from the National Institutes of Health, U. S. Public Health Service.

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