Taxonomic Characteristics of So-called 'Form 2 Mycobacteria'

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SUMMARY

Two strains of rapidly growing spore-forming bacteria were obtained from cultures of *Mycobacterium tuberculosis* and identified as so-called 'form 2 mycobacteria' (Csillag, 1961). The properties of these and of two similar strains received from Dr A. Csillag were investigated, and compared with those of NCTC strains of the genus *Bacillus*. All four 'form 2' strains were almost identical in character, and differed from *M. tuberculosis* in many respects, rendering it highly unlikely that they were genetically derived from the mycobacterial cultures in which they were found. They were identified as strains of *Bacillus licheniformis*. It is considered that 'form 2' organisms are not part of a complex mycobacterial life-cycle, as has been suggested, but are contaminants.

INTRODUCTION

The repeated isolation of rapidly growing bacteria from cultures of tubercle bacilli and 'anonymous' mycobacteria was reported by Csillag (1961). The chief characteristics of this observation were as follows. The mycobacterial cultures had to be incubated with aeration for 2-3 months for the growth to appear, and this growth consisted of rods which were not acid-fast, which stained variably by Gram's method and possessed endospores. Once manifest, these organisms grew readily on ordinary nutrient agar within a day or two. It was clear that the properties of these organisms differed greatly from those of the mycobacterial cultures in which they were found. On the other hand, they had some characteristics of bacteria of the genus *Bacillus*. Contamination was considered by Csillag as an explanation of the findings, but it was held that the precautions taken against it, the failure of the growths to appear in the controls, and the properties of the growths themselves rendered this explanation very unlikely. Csillag therefore concluded that they were actually derived from the mycobacteria present and represented an alternative form of existence of these organisms. For convenience of description she gave the name 'form 2' to the non-acid-fast growths, 'form 1' denoting the acid-fast organisms in this terminology. Further accounts of the emergence of form 2 strains in mycobacterial cultures growing on autoclaved Löwenstein-Jensen medium, and of their properties, have been published more recently (Csillag, 1962, 1963*a*, *b*, 1964*a*, *b*).

In view of the fundamental importance of these conclusions I tried to confirm the emergence of form 2 strains under the conditions described, and to assess their properties and significance. While it has not been possible to achieve consistent

isolation of such organisms, a few have been obtained. It is the purpose of this paper to describe the isolation of two of them and to outline their characteristics and those of two of Dr Csillag's own strains.

METHODS

Bacterial strains

Mycobacteria. Stock cultures: *Mycobacterium tuberculosis* H37RV ($\times 4$) (see below for the meaning of ($\times 4$), etc.) and H37Ra ($\times 2$); *M. bovis* NCTC9320, and a BCG strain obtained from Messrs Glaxo Ltd., Greenford, Middlesex ($\times 4$); *M. microti* ('vole bacillus') NCTC8710 ($\times 2$); *M. kansas* ('photochromogens'), 8 strains, nos. c12 ($\times 2$), 120, 335 ($\times 2$), 1640, 4619 ($\times 2$), 4777 ($\times 2$), 8841 ($\times 2$), 'Price' ($\times 2$) (these were obtained from Dr W. Pagel, Clare Hall Hospital, Barnet, with the exception of strain 'Price', obtained from Dr R. W. Riddell, Brompton Hospital, London). In the experiment described below for the isolation of form 2 bacteria, one culture of each strain was used, except as indicated above by the figure in parentheses, e.g. 'H37RV ($\times 4$)', which indicates that four cultures of the H37RV strain were used in that experiment.

Recent isolates of Mycobacterium tuberculosis. These consisted of 29 cultures from 25 strains, 4 of the strains being used in duplicate. These strains had been collected from the clinical laboratory over several months and were from the sputa of 25 untreated cases of pulmonary tuberculosis; all were isoniazid-sensitive and were nicotinic acid-positive by the method of Gutierrez-Vazquez (1958). Thirteen of the strains were the primary isolates themselves which had been incubated for 4–10 weeks from first appearance of growth, and stored at room temperature for periods between 1 and 16 weeks. The remainder were first or second subcultures of primary isolates.

Bacillus species. *B. licheniformis*, 3 strains (NCTC 6346, 7589, 8233); *B. subtilis*, 2 strains (NCTC 3610, 8236); *B. pumilus*, 2 strains (NCTC 7576, 8241); *B. cereus*, 1 strain (NCTC 7587).

Form-2 organisms. For convenience and clarity, the different form 2 strains referred to in this paper are designated with the prefix 'FT'. Two strains were received on agar slopes from Dr A. Csillag: one of them, strain FT1, had been found in a culture of *Mycobacterium tuberculosis*, no. I1413, from a British patient, and strain FT2 was from a similar culture, no. 59416, from an Indian patient.

General cultural methods

Mycobacterial cultures. Primary isolations of the strains of *Mycobacterium tuberculosis* from sputum were made by the trisodium phosphate method (Peizer, Chaves & Widlock, 1954), the treated deposits being grown on Löwenstein-Jensen medium (Mackie and McCartney's *Handbook*, 1960) without potato starch (Jensen, 1955) dispensed in 3 ml. amounts in $\frac{1}{2}$ oz. screw-capped bottles. Batches of the same medium without malachite green were also used. Strains of mycobacteria were maintained on Löwenstein-Jensen medium and were subcultured at intervals of a few months.

Bacillus species and form 2 cultures. The dried cultures received from the National Collection of Type Cultures were reconstituted with broth, and a loopful plated on nutrient agar. After incubation for 2 days a single representative colony was sub-

cultured on a nutrient agar slope (the primary culture), which was incubated for 3 days at 37° with the screw cap loose to encourage sporing, and then kept at room temperature indefinitely with the cap screwed tight. A single subculture from each primary culture was made on a nutrient agar slope treated similarly (secondary culture) and all the subcultures needed for the present work were made from the secondary cultures; the primary cultures were held in reserve in case of possible contamination of the secondary cultures. The nutrient agar slopes of the two form 2 strains (FT 1, FT 2) received from Dr Csillag were treated as primary cultures. After subcultivation of the growth from the slope and the condensation water to nutrient agar plates to test for purity, a secondary culture was made from each slope and used for all further tests. The two strains of form 2 bacteria (FT 3, FT 4) isolated during this work were subcultured to nutrient agar plates to test purity, and a single colony from each used to obtain primary and secondary cultures on nutrient agar slopes as described. Nutrient agar plates used for testing purity were incubated for 1 week.

The nutrient broth used was made from meat extract and peptone (Oxoid no. 2, Oxo Ltd., London), dispensed in 10 ml. amounts in $\frac{1}{2}$ oz. screw-capped bottles; nutrient agar consisted of the same medium solidified with 1.5% (w/v) agar, dispensed in slopes in similar bottles, or in plates. Blood agar was made by adding about 7% (v/v) oxalated horse blood (Wellcome Laboratories, Beckenham, Kent) to nutrient agar, previously melted and cooled to 50°. Blood agar plates were of the usual ‘sandwich’ pattern (*Mackie and McCartney’s Handbook*, 1960). Except as stated below, media were sterilized by autoclaving at 120° for 15 min. Incubation was aerobic at 37°, except where otherwise indicated.

Special differential tests

Special differential tests were used for the purpose of discovering similarities or otherwise between the four form 2 strains and the three species of *Bacillus* on the one hand, and the species *Mycobacterium tuberculosis* on the other. Many of the tests used were identical with or based on those described by Knight & Proom (1950) in a paper on the physiological characteristics of the *Bacillus* genus. Except where otherwise specified, the media used in these tests were inoculated from the condensation water of the stock slopes of the four strains of form 2 organisms, the three NCTC strains of *B. licheniformis*, and, where necessary for comparison, the other *Bacillus* strains. The minimum amount of condensation water held by a 3 mm. platinum loop was used. Any culture which showed growth was subcultured on a nutrient agar plate, incubated for 3 days and identified by its colonial and microscopic morphology. In a few tests inocula of mycobacteria were used: surface growth from a 4-week-old Löwenstein–Jensen slope was homogenized in sterile distilled water with the aid of glass beads as described by Csillag (1962), and a loopful of the homogenate used as inoculum.

Growth at different temperatures. Cultures were made in nutrient broth and incubated at 45°, 37° and room temperature (18–20°). A thermostatically controlled water bath was used for incubation at 45°, the cultures being totally immersed in it. The cultures were examined daily up to 3 days, and the amount of growth (turbidity and pellicle formation) recorded.

Motility. Overnight broth cultures at 37° were examined microscopically by a hanging-drop preparation.

Anaerobiosis. Nutrient agar plates were inoculated in duplicate, and one plate of each pair incubated up to 3 days anaerobically in a McIntosh and Fildes jar. The other plate was incubated aerobically for comparison.

Growth in the presence of 4% sodium chloride. Parallel cultures were made in nutrient broth with and without 4% NaCl (w/v) and incubated for 2 days. In addition, Dubos-Davis fluid medium (*Mackie and McCartney's Handbook*, 1960) was made up with and without 4% NaCl (w/v) and distributed in 10 ml. amounts in $\frac{1}{2}$ oz. screw-capped bottles. These were inoculated with the four FT strains and the three *Bacillus licheniformis* strains, and with 6 strains of *Mycobacterium tuberculosis* (the H37Rv strain and 5 strains recently isolated from sputum). The mycobacterial cultures were incubated for 28 days (when all the control cultures without added NaCl showed good growth) and the remainder for 2 days.

Voges-Proskauer reaction, nitrate reduction and citrate utilization. Cultures in M.R.V.P. medium (Oxoid) were incubated for 2 days and tested for the formation of acetylmethylcarbinol (*Mackie and McCartney's Handbook*, 1960). Reduction of nitrate to nitrite was tested by the method of Skerman (1959): samples were taken after 1, 2 and 3 days of incubation and tested for nitrite formation by a spot test on a white tile with starch + iodide indicator. Cultures were also tested for the production of gas from nitrate under anaerobic conditions (Gibson, 1944). Citrate utilization was tested by inoculating Koser's citrate medium (Oxoid), incubating for 2 days, and observing the development of turbidity; a loopful of any growth thus seen was subcultured to a new bottle of the same medium, and citrate utilization was assumed to occur only when the second subculture also showed growth of the strain under test. In these tests, the NCTC strains of *Bacillus subtilis* and *B. pumilus* were also used.

Action on carbohydrates. The ammonia basal medium of Knight & Proom (1950) was used: this medium contains ammonium phosphate as the only source of nitrogen, and whichever carbohydrate is added constitutes the only source of carbon. It was found convenient to adopt the following modifications: the medium was adjusted to pH 7.4 and phenol red 0.01% (w/v) and agar 1.5% (w/v) were added. Stock solutions of the carbohydrates, 10% (w/v) in distilled water, were sterilized by Seitz filtration and were added to the melted basal medium to a final concentration of 1% (w/v). The medium was dispensed as slopes in $\frac{1}{2}$ oz. screw-capped bottles. The bottles were incubated for 3 days; surface growth indicated utilization of the carbohydrate tested, and yellow coloration of the medium indicated the development of an acid reaction. In addition to the form 2 strains and *Bacillus licheniformis* strains, the *B. subtilis*, *B. pumilus* and *B. cereus* strains were also tested in order to confirm that the results obtained with the medium were similar to those described by Knight & Proom (1950). None of the organisms grew on the basal medium without the addition of carbohydrate. The formation of CO₂ gas from glucose under semi-anaerobic conditions was also tested for. The test was done as described by Gibson & Abd-el-Malek (1945) with the following modifications. The medium consisted of 5% (w/v) peptone (Oxoid) with 5% (w/v) glucose in distilled water, adjusted to pH 7.3, and distributed into U-tubes each having one closed limb which was completely filled with medium. The surface of the medium in the open limb was sealed after inoculation with a layer of paraffin wax 1 cm. deep. After incubation up to 5 days, the amount of bacterial growth and the size of any gas bubbles in the

closed limb were noted; shrinkage of the latter after the addition of alkali indicated their CO₂ content. *Bacillus subtilis* and *B. pumilus* strains were included in this part of the investigation.

Enzymic activities. (1) *Urease.* The rapid test for urease activity of Elek (1948) was used. The form 2 and NCTC culture of *Bacillus* species were grown for 2 days on nutrient agar, and a loopful of the growth used as the inoculum. The same 6 cultures of *Mycobacterium tuberculosis* referred to earlier in this section were also tested for urease production by this technique: a well-loaded loopful of the surface growth formed the inoculum.

(2) *Hydrolysis of starch, gelatin and egg albumen.* For starch hydrolysis a 5% (w/v) solution of soluble starch (AnalaR) in distilled water was sterilized by steaming for 1 hr on three successive days. Five drops of this solution were added with a Pasteur pipette to 10 ml. nutrient broth, which was then inoculated. After incubation for 2 days a few drops of the culture were placed on a white tile and Gram’s iodine solution added: failure to develop a blue-black coloration indicated complete hydrolysis of the starch. In cases of doubt, the culture was re-incubated for 2 days after the addition of 5 more drops of starch solution and re-tested. Negative controls were provided by the use of uninoculated starch broth incubated similarly, and of cultures inoculated with *Bacillus pumilus* (Knight & Proom, 1950). For gelatin hydrolysis bottles ($\frac{1}{2}$ oz.) containing 10 ml. nutrient broth solidified with 10% gelatin were inoculated and incubated at 37° up to 5 days. The cultures were tested daily by bringing them to 0–4° in a melting ice bath. Failure to solidify at this temperature indicated gelatinase action. Control uninoculated bottles of gelatin medium were tested in parallel. Egg albumen hydrolysis: liquefaction of Löwenstein–Jensen medium by form 2 cultures was confirmed, and compared with the three type strains of *B. licheniformis*, by inoculation on to slopes of this medium made up without malachite green, with incubation up to one week.

Sensitivity to chemotherapeutic agents. *Isoniazid sensitivity.* Dubos–Davis fluid medium (see above) containing isonicotinyl hydrazide 500 µg./ml. was used. Screw-capped $\frac{1}{2}$ oz. bottles containing 10 ml. of this medium were inoculated and incubated for 2 days. *Other drugs.* Sensitivity to penicillin, streptomycin, tetracycline, chloramphenicol, erythromycin and sulphadimidine was tested by the disc diffusion plate technique of Fairbrother & Martyn (1951) on the medium of Jewell & Pearmain (1954). The amount of each drug incorporated in the discs was such as to produce inhibition zones of 15–20 mm. diameter after overnight incubation on plates inoculated with the standard sensitive Oxford strain of *Staphylococcus aureus*.

Endospore characteristics. (1) *Preparation of spore suspensions.* The four strains of form 2 organisms and three *Bacillus licheniformis* strains were inoculated into $\frac{1}{2}$ oz. bottles of nutrient broth and incubated for 4 days with the caps of the bottles loose. The bottles were shaken vigorously once each day (with the cap tight) to break up the pellicles and to increase aeration. The contents of each bottle were homogenized in a sterile Griffith hand-operated glass grinder, and the bottles allowed to stand for 10 min. so that any coarse particles remaining could settle. The turbid supernatant fluid was separated and 3 ml. volumes stored in $\frac{1}{4}$ oz. screw-capped bottles at 4°. Microscopic examination of stained smears at this stage showed that the great majority of the organisms were in the spore form.

(2) *Heat resistance.* From each suspension before heating a standard loopful was taken and spread evenly over the surface of a nutrient agar plate. Samples (2 ml.) of each suspension were placed in $\frac{1}{4}$ oz. bijou bottles, which were totally immersed in a water bath already at the required temperature. The bottles were shaken gently during the first 5 min. of each period of heating to ensure the even distribution of heat through the suspension. After the chosen period of time had elapsed, a loopful of the heated suspension was spread on a plate of nutrient agar as in the case of the unheated control. All the plates were incubated overnight and the amounts of growth on each compared. The following temperature/time exposures were used: 70°/30 min., 80°/30 min., 90°/20 min., 100°/20 min.

Inhibition by malachite green in Löwenstein-Jensen medium. Several techniques were used to test the capacity of form 2 organisms and *Bacillus* strains to grow on Löwenstein-Jensen medium with and without malachite green; these will be described elsewhere. In general, assessments were made of the power of vegetative forms, unheated spores and spores heated at 80°/30 min. to initiate growth from inocula varying in size from about 10 up to 10⁶ viable organisms on Löwenstein-Jensen medium, with and without malachite green, dispensed in bottles or in plates.

Special differential tests. Smears were allowed to dry at room temperature and were heat-fixed. Gram, Ziehl-Neelsen and spore staining methods were done as described by Csillag (1961), except that spirit (90 %, v/v, ethanol in water) was used instead of acetone for decolorization in Gram's method.

RESULTS AND DISCUSSION

The isolation of form 2 bacteria

Fifty-six cultures of mycobacteria on Löwenstein-Jensen medium were used. They comprised 27 recent isolates of *Mycobacterium tuberculosis*, together with 27 stock cultures, 13 of these being *M. kansasii*. The origin and previous handling of these cultures is described above; at the time the experiment was started each culture bottle contained a well-developed surface growth. Initially, each culture was checked to ensure that the growth did consist of acid-fast bacilli, and it was then subcultured to nutrient agar to make sure that no form 2 or other organisms were already present. The cultures were then incubated for 20 weeks; they were aerated intermittently during this period by removing the screw cap of the bottle for a few seconds twice a week. Once every 2 weeks the surface growths were subcultured to nutrient agar plates to detect form 2 bacteria. From this experiment only two form 2 strains were obtained: one appeared at the 6th week of incubation with aeration, and the second at the 8th week. Both appeared in bottles containing primary isolates of *M. tuberculosis* obtained from the sputa of different patients, and their presence in the cultures was obvious even before subculture because the egg medium turned brown and became liquefied. Each gave a confluent growth on nutrient agar after overnight incubation. None of the remaining 54 cultures yielded growth on nutrient agar before being discarded after a total of at least 24 weeks' incubation (4-10 weeks preliminary, 20 weeks with aeration). Stock cultures were made and the two form 2 strains thus isolated were designated FT3 and FT4. Subcultures were sent to Dr A. Csillag: she confirmed that they were indeed form 2 organisms, and kindly sent me two of her strains for comparison. The characteristics

of these four form 2 strains were then investigated; since early findings suggested a strong resemblance to organisms of the genus *Bacillus*, NCTC strains of this genus (enumerated above) were tested in parallel.

The properties of form 2 bacteria

Morphology and staining reactions. All four strains consisted of rods which were fairly all consistent in thickness (about 0.8μ) but variable in length. Overnight cultures in broth or on the surface of nutrient agar showed mainly rods $5-7\mu$ in length. Long filaments were also seen; these on close inspection were streptobacillary in form, consisting of closely connected rods of the length indicated above.

Overnight cultures usually showed occasional spore-containing bacilli; at 2 days nearly all showed spores; and at later times the cultures consisted almost entirely of spores lying free or arranged in chains, with only a few bacillary bodies remaining. The appearances presented by spore-containing form 2 bacteria were those of *Bacillus* species of morphological group 1 (Smith, Gordon & Clark, 1946), in which the sporangium is only slightly swollen, or not swollen at all, with a thin-walled oval spore.

The growths were invariably not acid-fast. They were easily stained at room temperature by methylene blue or other simple stains, and under suitable conditions were Gram-positive. This was a variable and unpredictable property, however, and more often than not the organisms appeared to be Gram-negative. At times different rods in the same chain would show a different Gram-staining reaction. These morphological and staining properties of the form 2 strains, however, resembled closely those of the NCTC strains of *Bacillus licheniformis*, when the latter were grown under similar conditions and stained on the same slides.

Cultural characteristics. The aerobic growth of these form 2 strains in nutrient broth and on nutrient agar was very rapid. After overnight incubation at 37° in broth, a firm surface pellicle was formed, with moderate general turbidity. On microscopic examination in fresh preparation, all four strains showed very active motility. On nutrient agar large colonies (3-4 mm. diameter) were formed after incubation for 24 hr. The appearance of these colonies was extremely variable, and very dependent on the type, or even batch, of medium used, as well as on other unpredictable factors in the cultural conditions. In general, early colonies were grey, semi-translucent and faintly greenish in hue, and rhizoid in character. Later, particularly on media containing blood or serum, small patches of mucoid growth appeared, resembling beads of slightly milky fluid resting on the rhizoid growth. At times the growth was entirely mucoid, but usually the mixed mucoid + rhizoid appearance was seen; this is well illustrated in fig. 5, plate 2, of Dr Csillag's paper (Csillag, 1961). Growths on protein-containing medium, especially on Löwenstein-Jensen medium without malachite green, became pigmented after a few days of incubation, showing a strong reddish purple colour. Surface growths from heavy inocula on blood agar, taken freshly from the incubator, had the strong, sour, musty and offensive smell characteristic of some species of the genus *Bacillus*, and wide zones of haemolysis, with greenish staining, were formed round the colonies. No diffusible pigment was formed in the absence of blood. In all these general cultural characteristics, the form 2 organisms were indistinguishable from the NCTC strains

of *Bacillus licheniformis*, and were quite unlike mycobacteria in general, and *Mycobacterium tuberculosis* in particular.

The results of the specific differential tests used in investigating these organisms, together with some of those already mentioned, are set out in Table 1. Those tests marked with an asterisk were used by Knight & Proom (1950) for characterizing and distinguishing various species of the genus *Bacillus*. Most of the properties of the form 2 strains were re-tested after repeated single-colony subcultures, and also after the stock cultures had been kept for several months, and were found to be unaltered.

Table 1. *A summary of the characteristics of four form 2 strains (FT1 to 4) and a comparison of their properties with those of three strains of Bacillus licheniformis (NCTC 6346, 7589, 8233) and with those of Mycobacterium tuberculosis*

	NCTC 6346	NCTC 7589	NCTC 8233	FT 1	FT 2	FT 3	FT 4	<i>M. tuberculosis</i>
Growth in 24 hr in broth at 37°	+	+	+	+	+	+	+	—
*Growth at 18°	+	+	+	+	+	+	+	—
*Growth at 45°	+	+	+	+	+	+	+	—
*Growth in 4 % NaCl medium	+	+	+	+	+	+	+	—
*Growth under anaerobiosis	+	+	+	+	+	+	+	—
*Growth in synthetic medium with glucose	+	+	+	+	+	+	+	—
*Citrate utilisation	+	+	+	+	+	+	+	—
Colonies 3–4 mm. diam. formed in 24 hr	+	+	+	+	+	+	+	—
Haemolysis on blood agar	+	+	+	+	+	+	+	—
Purple-red pigment formation	+	+	+	+	+	+	+	—
Surface pellicle in 24 hr	+	+	+	+	+	+	+	—
Acid-fast	—	—	—	—	—	—	—	+
*Rods 0.8 μ diam.	+	+	+	+	+	+	+	—
Variable Gram reaction	+	+	+	+	+	+	+	—
*Motile	+	+	+	+	+	+	+	—
*Endospore formation	+	+	+	+	+	+	+	—
*Nitrate reduction	+	+	+	+	+	+	+	+
*Voges-Proskauer reaction	+	+	+	+	+	+	+	N
*Acid from xylose	+	+	+	+	+	+	+	—
*Acid from arabinose	+	+	+	+	+	+	+	—
Acid from mannitol	+	+	+	+	+	+	+	—
*Gibson-Abd-el-Malek reaction	+	+	+	+	+	+	+	—
*Urease	—	—	—	—	—	—	—	+
*Starch hydrolysis	+	+	+	+	+	+	+	N
*Gelatine liquefaction	+	+	+	+	+	+	+	—
Egg albumen liquefaction	+	+	+	+	+	+	+	—
Sensitive to isoniazid	—	—	—	—	—	—	—	+
Sensitive to penicillin	—	—	+	+	—	+	+	—
Sensitive to streptomycin	+	+	N	+	+	+	+	+
Sensitive to tetracycline	+	+	N	+	+	+	+	+
Sensitive to chloramphenicol	+	+	N	+	+	+	+	+
Sensitive to erythromycin	+	+	N	+	+	—	+	—
Sensitive to sulphadimidine	+	+	N	+	+	+	+	+
Sensitive to malachite green	+	+	+	+	+	+	+	—

* Differential characteristics of *B. licheniformis* (Knight & Proom, 1950).

+, Indicates possession of the property described; —, indicates non-possession of the property described; N, indicates strain not tested, or no reference in literature.

Where valid comparison is possible, the characteristics of *Mycobacterium tuberculosis* are also included in Table 1. These have been taken from *Topley & Wilson's Principles* (1964) and Soltys, St Hill & Ansell (1952), supplemented by reference to other authors cited in the text below, or by experimental work forming part of the present work. Several of the results recorded in Table 1 need no discussion at this stage; others need further comment and are discussed below.

Effect of cultural conditions. The form 2 strains are clearly able to grow in a wide variety of nutritional environments and of conditions of incubation, in which they differ from *Mycobacterium tuberculosis*. Of particular note is their power to grow under strictly anaerobic conditions, in which their growth was only slightly less than when grown aerobically. This is in very marked contrast to *M. tuberculosis*, which is killed by anaerobic incubation (Loebel, Shorr & Richardson, 1933; Guy, Raffel & Clifton, 1954). No literature reference was found to the effect of 4% NaCl on mycobacteria. Six strains of *M. tuberculosis* were therefore tested in fluid medium containing 4% NaCl; all six strains did not grow. The form 2 and *Bacillus licheniformis* strains showed equally good growth in this medium and in nutrient broth whether with added NaCl or not. This property was found by Knight & Proom (1950) to be possessed by almost every species in the genus *Bacillus* which they tested.

Biochemical and enzymic activities. Formation of nitrite from nitrate occurred with the form 2 strains, and with *Bacillus licheniformis* and *B. subtilis* but not with *B. pumilus* in agreement with Knight & Proom (1950). This was one of the few characters which form 2 strains had in common with mycobacteria (Virtanen, 1960; Hedgecock & Costello, 1962); however, the formation of nitrogen gas from nitrate (Gibson, 1944) and of carbon dioxide from 5% glucose (Gibson & Abd-el-Malek, 1945), which was found with both form 2 and *B. licheniformis* strains, cannot occur with the mycobacteria because of their requirement of aerobic growth.

Utilization of citrate as sole carbon source. This was found with all four form 2 strains. Although citrate enhances the growth of *Mycobacterium tuberculosis*, it is apparently not utilized as sole carbon source (Edson, 1951). The form 2 strains also grew well, with acid production, from small inocula on the defined medium with ammonia as the sole source of nitrogen, and glucose, xylose, arabinose or mannitol as carbon source. The action of *Bacillus licheniformis* on the first three of these carbohydrates was described by Knight & Proom (1950) and on mannitol by Cowan & Steel (1961). Although large inocula of certain strains of *M. tuberculosis* can initiate growth on similar defined media, small inocula cannot do so without the addition of more complex organic materials, especially in the case of recently isolated strains (Middlebrook & Dubos, 1958). Although glycerol and glucose can be assimilated by *M. tuberculosis*, xylose, arabinose and mannitol cannot (Edson, 1951; *Topley & Wilson's Principles*, 1964).

Urease formation. Urease is formed by mycobacteria with the exception of *Mycobacterium avium* and certain related strains (Pawlicki, Hertz & Green, 1963; Oka & Yamaguchi, 1963), and all 6 strains of *M. tuberculosis* tested by the method of Elek (1948) were positive; the form 2 strains and *Bacillus* strains were negative. Knight & Proom (1950) also found these *Bacillus* species to be urease-negative.

Hydrolysis of starch, gelatin and more complex proteins. These properties are characteristic of *Bacillus licheniformis* (Knight & Proom, 1950) and were found to be possessed by the form 2 strains. It is common knowledge that the mycobacteria do

not digest Löwenstein-Jensen medium, or other coagulated egg media, nor do they liquefy gelatine (Skerman, 1959). There is no information about the action of *Mycobacterium tuberculosis* on starch in the literature references examined and this property was not investigated.

The effect of antibacterial agents. The form 2 strains differed from *Mycobacterium tuberculosis* in being highly resistant to isoniazid, and, in the case of three of them, in being penicillin-sensitive. In the cases of the other five drugs tested, the form 2 strains did not differ significantly from the *Bacillus licheniformis* strains in their sensitivity patterns.

The reasons for testing the capacity of form 2 strains to grow on Löwenstein-Jensen medium with and without malachite green, and the detailed results, will be described elsewhere. In general, the spores of the form 2 strains did not initiate growth on this medium containing malachite green; the vegetative forms did so in a rather capricious way, with the exception of strain FT 1, which did not yield growth after incubation for 12 days from inocula ranging from 10 to 10^4 viable organisms. However, small inocula of the spores or vegetative forms of all four strains grew as readily on Löwenstein-Jensen medium without malachite green as on blood agar. The *Bacillus licheniformis* cultures, tested in parallel, showed very similar patterns of inhibition by malachite green. These characteristics contrast with those of *Mycobacterium tuberculosis*, since Löwenstein-Jensen medium containing malachite green is the standard medium for the cultivation of small numbers of tubercle bacilli.

Heat resistance of the spores. The spores of the form 2 strains and the *Bacillus licheniformis* strains remained viable after exposure to moist heat at 90° for 20 min., but were not viable after 20 min. at 100°.

Interpretation of the findings

In so far as the results reported here on the properties of form 2 strains confirm those reported by Csillag (1961, 1962, 1963 *a*, *b*), no serious differences have been found. All four form 2 strains appear to be of the 'large colony' type which she considered to be the final form of these organisms when obtained from the majority of cultures of *Mycobacterium tuberculosis* (Csillag, 1962). There is agreement that microscopically they appear as rather coarse (0.8μ thick) rods and filaments, with extremely variable Gram-staining reaction, and form endospores; both accounts of the degree of heat resistance of the latter are consistent (Csillag, 1962). All the form 2 strains grow rapidly on nutrient agar and are facultatively anaerobic. There can be no question therefore that organisms of this kind can be isolated from mycobacterial cultures handled in the way described by Csillag; only the interpretation of the findings is in doubt.

In any experimental situation in which bacterial cultures are found to show altered characteristics, there are two possibilities: either the new properties have been acquired by the parent stock (in which case one is working with a population genetically derived from the parent) or the characters are those of an extraneous and unrelated species, contamination being the explanation of the findings. When a variant can be isolated regularly under defined conditions, when only one or a few of its properties are found to be changed, and when by a considerable number of other tests the new population can be identified with the parent, contamination

need not be considered. The situation is different, however, when several characteristics appear to have altered simultaneously. There are no definite rules about the number of characteristics by which a variant may differ from its parent and still be accepted as such rather than as another species, but in general terms, the greater its divergence from the parent, the greater is the likelihood that another species is being investigated (Hilson & Elek, 1959). When the new population under investigation can be identified with a well-defined (different) bacterial species, especially when the latter is well known to be a common laboratory contaminant, then the probability of contamination is overwhelming. Very weighty experimental evidence to the contrary is needed before the alternative hypothesis of a new and unparalleled genetic change may be accepted.

The four strains of form 2 bacteria studied differ from *Mycobacterium tuberculosis* in many ways, showing characters gained (such as motility, spore formation, facultative anaerobiosis) and lost (such as acid-fastness, urease activity). These properties appear to be genetically determined, since they are stable after serial subculture and after storage. In general terms the mathematical probability of the appearance in a bacterial population of a variant showing multiple genetic changes is the geometric sum of the probabilities for each individual change. It could, however, be argued that the simultaneous appearance of several altered characters might be mediated through a smaller number of actual genetic changes, with linkage of metabolic systems as the explanation of the numerous phenotypic differences; the mathematical probability of an event of this kind taking place would of course be less minute. The nearest analogy is perhaps provided by the appearance of the resistance transfer factor in *Shigella* species (Nakaya, Nakamura & Murata, 1960; Watanabe & Fukasawa, 1960) which mediates the simultaneous development of resistance to streptomycin, chloramphenicol, tetracycline and sulphonamide. This phenomenon, however, provides only a very remote analogy to the one under discussion; and there is no other evidence in the field of bacterial genetics to indicate that changes in so many unrelated structural and functional characters could be mediated by one, or very few, mutational steps. A large number of independent genetic alterations would be needed, and these could be found only in a bacterial population of astronomical size initially, or after an astronomical number of generations. However, form 2 organisms do not appear in mycobacterial cultures until various periods after the active growth phase is over, when there is little further multiplication. It is therefore highly unlikely on genetic grounds that form 2 bacteria are really the progeny of the mycobacterial population in which they appear.

On the other hand, all four form 2 strains possess in common more than thirty characteristics shown by NCTC strains of *Bacillus licheniformis* tested in parallel, the only difference of note being in respect of penicillin sensitivity, in which the *B. licheniformis* strains themselves were not consistent. Furthermore, all four form 2 strains possessed all the criteria for the identification of the species *B. licheniformis* described by Knight & Proom (1950). Therefore, whether the identity of these strains is determined on the time-honoured basis of giving greater weight to certain properties (e.g. growth rate, endospore formation) than to others, or on the Adansonian basis, more favoured at present, of an assessment of the sum total of properties held in common (Sneath, 1962), the answer is clearly the same: all four are examples of *B. licheniformis*.

This conclusion differs fundamentally from that of Csillag (1961, 1963*a, b*), who, though noting the general resemblance between form 2 bacteria and organisms of the genus *Bacillus*, did not classify them in this group but suggested that they should be placed in the Order Actinomycetales, with closest resemblance to the Actinomycetaceae. This would make them more nearly related to the mycobacteria than if they were placed in the genus *Bacillus*. The criteria on which this decision was reached were almost entirely morphological, being based on a study of the macroscopic and microscopic appearances of slide-cultures on glycerol agar sampled at intervals up to 6 weeks; no investigation of metabolic activities was made, apart from a comparison of the effect of aerobiosis and anaerobiosis on the cultures. It should be noted, however, that only two reasons were given for not including these organisms in the genus *Bacillus*: (1) the high incidence of Gram-negative forms; (2) the formation of 'coccoid elements' apparently capable of continued multiplication by binary fission. In fact, in respect of variability of staining, these organisms cannot be distinguished from the NCTC strains of *Bacillus licheniformis* and they are Gram-positive in the taxonomic sense. The significance of the second reason (binary fission of 'coccoid elements') is somewhat obscure. It was assumed that the coccoid elements were formed in the same way as the 'fragmentation spores' of the Actinomycetaceae, and were analogous to them. This assumption was based on observations made of different cultures incubated for various periods, an approach that renders morphological deduction difficult. Leaving aside such difficulties of interpretation, the morphology of species belonging to the genus *Bacillus* and to the Actinomycetales is extremely variable and dependent on the minutiae of medium composition and conditions of culture (Knight & Proom, 1950; Waksman, 1957). No direct comparison of form 2 strains with *Bacillus* strains on glycerol agar was reported. It is conceivable that coccoid elements might also have appeared in the latter under such conditions. On the other hand, Csillag mentions three characters of the form 2 bacteria which are inconsistent with classification within the Actinomycetaceae: endospore formation, absence of true branching, and lateness of formation of 'coccoid fragmentation spores'. The first of these three is perhaps the most important, since the endospores are heat resistant, a property confined to the spores of the genera *Bacillus* and *Clostridium*.

The phenomenon of 'dimorphism' in fungi was suggested as an analogy to the appearance of form 2 bacteria in mycobacterial cultures (Csillag, 1961). This term dimorphism is applied to the change in morphology which is manifested by certain fungal species in response to a defined environmental stimulus, and which is reversed when the stimulus is removed. The phenomenon is exemplified by the hypha-to-yeast change induced in *Histoplasma* or *Blastomyces* by raising the temperature of incubation to 37° (Conant *et al.* 1944), or the similar change induced by the addition of carbon dioxide to anaerobic cultures of *Mucor* (Bartnicki-Garcia, 1963). However, there does not appear to be any real resemblance between the form 2 phenomenon and dimorphism. No reversion to the original acid-fast form has been described. Nor is there any parallel in dimorphism to the widespread metabolic changes shown by form 2 organisms when compared with the mycobacteria from which they are supposed to be derived. In *Mucor*, for example, alternation in morphology induced by carbon dioxide is accompanied by little or no change in major physiological processes (Bartnicki-Garcia, 1963).

The evidence presented above shows, first, that the possibility that form 2 bacteria may be genetically derived from mycobacteria must be considered as very remote, and, secondly, that they can be identified as *Bacillus licheniformis*, a spore-bearing species commonly found as a laboratory contaminant. There is therefore a strong *a priori* suspicion that contamination is the explanation of the form 2 phenomenon. In postulating a different origin for these organisms, in the manner of Csillag, the burden of proof lies in showing that they can be isolated regularly from mycobacteria under defined conditions, and that their rate of isolation from mycobacterial cultures is significantly greater than from properly designed controls kept under identical conditions. However, in work to be published in detail elsewhere, I did not find it possible to reproduce the regularity of isolation of form 2 bacteria described by Csillag (1961). I also found that inocula composed of a few spores of form 2 organisms, or of *Bacillus licheniformis* (i.e. inocula of a size which would be expected in random contamination) would not grow on control medium of the kind used by Csillag, whereas they did so readily on the same medium modified by the previous growth of mycobacteria on its surface. The validity of this finding, made by the use of spores freed from vegetative forms by heating, has been contested by Csillag (1964*b*), but my work with unheated spores and with vegetative forms has essentially confirmed it. This being the case, the control cultures of Csillag (1961) were unsatisfactory, since they could not reveal the presence of minimal contamination of this kind, which would, however, be made manifest by the mycobacterial growths in the test cultures. Hence it would appear that the contaminants were derived from the mycobacterial inocula.

It is clear that there is no agreement at present as to the interpretation to be placed upon the form 2 phenomenon, and it is therefore highly desirable that other workers should investigate the problem; however, my belief is that it is unnecessary to postulate a complex life-cycle for mycobacteria, and that the contamination of mycobacterial cultures by extraneous spore-bearing organisms of the genus *Bacillus* provides the entire explanation.

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