Growth of a Form 2 *Mycobacterium* and Various *Bacillus* Species on Löwenstein-Jensen Medium

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(Received 18 June 1963)

SUMMARY

Vegetative organisms of a Form 2 strain of *Mycobacterium tuberculosis* were inoculated on to Löwenstein-Jensen medium containing malachite green. The multiplication of the organisms was slightly delayed by the presence of the dye, but after a few days growth occurred, even when only a few organisms were used as inoculum. Stimulation of growth of Form 2 mycobacteria by the presence of Form 1 mycobacteria was not observed. Spores of *Bacillus subtilis* and *B. licheniformis* grew on Löwenstein-Jensen medium. Organisms other than *Bacillus* were usually obtained from Löwenstein-Jensen medium slopes deliberately exposed to airborne contamination, but have never been found in experiments in which *M. tuberculosis* Form 2 organisms were isolated. These results establish the validity of the uninoculated control slopes of Löwenstein-Jensen medium used in all previous experiments in which Form 2 mycobacteria were obtained from Form 1 mycobacteria.

INTRODUCTION

Methods of obtaining rapidly growing spore-forming non-acid-fast rods (Form 2 mycobacteria) from intermittently aerated cultures of *Mycobacterium tuberculosis* (Form 1 mycobacteria) on Löwenstein-Jensen medium were described in previous publications (Csillag, 1961, 1962, 1963*b*). Most of the test cultures were inoculated from old (6 weeks or more) Löwenstein-Jensen medium cultures and were incubated for several months, during which period air was let into the cultures usually once or twice a week and subcultivations were made on to nutrient agar plates. The presence of Form 2 mycobacteria was shown by discoloration of the Löwenstein-Jensen medium and by growth on the nutrient agar. In all of these experiments several uninoculated slopes of the same batch of Löwenstein-Jensen medium were subjected to the same aeration and subcultivation procedures: none showed any evidence of bacterial growth as indicated by discoloration of the medium, the presence of colonies or growth on subculture on nutrient agar. These findings were part of the evidence that Form 2 organisms were derived from Form 1 mycobacteria and were neither medium nor air contaminants. However, Hilson (to be published) has suggested that Form 2 mycobacteria arise from contaminatory *Bacillus* organisms present in small numbers in the medium or which gain access to the cultures during aeration. His contention is that the contaminants are inhibited by the malachite green in the uninoculated Löwenstein-Jensen medium, but are able to grow in

cultures which contain Form 1 mycobacteria because of destruction of the dye by the Form 1 organisms. The supposition that Form 2 mycobacteria arose from medium contaminants has been shown to be unlikely since they were found to develop in Form 1 cultures on autoclaved medium at a similar rate as on inspissated medium (Csillag, 1962). The possibility that Form 2 organisms arose from airborne contamination has now been further investigated and the results are presented here.

METHODS

Organisms. Mycobacterium tuberculosis. (1) Strain 11413, isolated from the sputum of a British patient with pulmonary tuberculosis (Csillag, 1963*a*), was maintained on Löwenstein–Jensen medium for about 3 years and a 5-week culture was used in the present experiment. (2) A Form 2 strain, isolated from a freeze-dried culture of strain 11413 (Csillag, 1963*b*) was maintained as described earlier (Csillag, 1963*a*), and a 10-week culture on nutrient agar served as the initial culture. *Bacillus licheniformis* (NCTC 1158) and *B. subtilis* (NCTC 2591) were grown on nutrient agar in $\frac{1}{2}$ oz. screw-capped bottles with slightly loosened caps for 3 days at 37°. The caps were then closed and the cultures were kept at room temperature for 2 years (initial cultures).

Media. (1) Löwenstein–Jensen medium (Mackie & McCartney, 1960) without potato starch (Jensen, 1955) and the same medium without malachite green were dispensed in $\frac{1}{2}$ oz. screw-capped bottles. (2) Nutrient agar was prepared by adding 1.4% (w/v) agar to meat-extract + peptone broth (Oxoid No. 2, Oxo Ltd., London) and was used either as slopes in $\frac{1}{2}$ oz. screw-capped bottles or as plates. (3) Hartley's digest nutrient broth (Mackie & McCartney, 1960) was used as the diluent for viable counts. All media were incubated for 3 days at 37° before use, as a test of their sterility. All cultures were incubated at 37° unless otherwise stated.

RESULTS

Growth of vegetative organisms of Form 2 mycobacteria on Löwenstein–Jensen medium. An inoculum containing only a few vegetative organisms of the Form 2 strain of *Mycobacterium tuberculosis* was prepared in the following way. The initial Form 2 culture was plated on nutrient agar; after incubation for 2 days, a suspension, prepared from an isolated colony, was inoculated into nutrient broth and was shaken for 24 hr. Serial ten-fold dilutions of this broth culture were inoculated in 0.02 ml. volumes on two slopes of Löwenstein–Jensen medium + malachite green and on the surface of two nutrient agar plates. The nutrient agar plates were incubated for 1 day, and the colonies then counted. The Löwenstein–Jensen medium cultures were incubated for 4 days, and representative samples of surface growth and of condensation water then subcultivated to further nutrient agar plates which were incubated for 1 day. The 10^{-6} dilution yielded 3 and 9 colonies on the nutrient agar plates. The Löwenstein–Jensen medium slopes inoculated with this dilution turned dark green after incubation for 2 days and colonies were then visible on them; subculture to nutrient agar yielded a heavy growth of Form 2 colonies. The 10^{-7} dilution yielded 1 and 0 colonies on nutrient agar and the Löwenstein–Jensen medium slopes remained unchanged and were sterile on subculture.

The same experiment was repeated with the following modifications. The 10^{-6} dilution only, containing 3–10 organisms capable of yielding colonies on nutrient agar plates, was inoculated on to 6 nutrient agar slopes, on to 6 slopes of Löwenstein–Jensen medium + malachite green and on to 6 slopes of the same medium on which were growing 5-week cultures of Form 1 *Mycobacterium tuberculosis*. On the nutrient agar slopes colonies appeared after incubation for 1 day. The Löwenstein–Jensen medium slopes (without Form 1 growth) became discoloured and colonies appeared on them after incubation for 2–4 days. Brownish discoloration appeared in the Löwenstein–Jensen Form 1 cultures after incubation for 3 days. Nutrient agar plates inoculated from all of the cultures yielded heavy growth of Form 2 mycobacteria.

Growth of spores of various species of Bacillus on Löwenstein–Jensen medium. Inocula containing free endospores of some *Bacillus* species were prepared in the following way. Smears prepared from 2-year old cultures of *Bacillus subtilis* and *B. licheniformis* showed numerous free endospores and only occasional vegetative forms. A moist sterile loop was placed within the culture bottle but without touching the medium. The bottle was then tapped to dislodge spores on to the loop. Loops, thus inoculated, were spread on 4 slopes of Löwenstein–Jensen medium + malachite green. After incubation for 2 days the medium became yellow and then pink on the following day. Growth was visible after incubation for 2 days and subculture to nutrient agar yielded a heavy growth of *Bacillus* organisms.

Table 1. *Airborne contaminants obtained on various media*

Twenty slopes of each type of medium were exposed to airborne contamination overnight in the laboratory and resulting growth was identified after subculture to nutrient agar.

Organisms obtained on subculture to nutrient agar	Medium		
	Löwenstein– Jensen + malachite green	Löwenstein– Jensen without malachite green	
		Number of slopes	Nutrient agar
Gram-positive, spore- bearing rods	1	2	1
Other organisms	6	7	9
Sterile	13	11	10

Growth of airborne contaminants on Löwenstein–Jensen medium. Twenty slopes of Löwenstein–Jensen medium + malachite green, 20 slopes of the same medium without malachite green and 20 slopes of nutrient agar were placed in a rack in a random order. The screw-caps were taken off and the media exposed overnight to the air in the same laboratory where Form 2 mycobacteria had been, and were being, isolated from Form 1 strains. The screw-caps were then replaced and the surface of the slopes washed with their own condensation water. After incubation for 2 days, representative samples, taken from the surface of the slopes and from the condensation waters, were plated on nutrient agar and incubated for 1 day. The results obtained on the nutrient agar plates are given in Table 1. About half

of the slopes of each type of medium yielded growth. Gram-positive spore-bearing rods were obtained from 1 Löwenstein-Jensen slope + malachite green, from 2 Löwenstein-Jensen medium slopes without malachite green and from 1 nutrient agar slope. The remainder of the positive cultures yielded various other organisms.

DISCUSSION

The findings reported here may be considered in the light of the suggestion that Form 2 mycobacteria arise from contaminants unable to grow on Löwenstein-Jensen medium + malachite green unless the medium has been conditioned by the previous growth of a Form 1 strain (which destroys the dye). (1) The multiplication of the vegetative organisms of the Form 2 strain investigated was slightly delayed by the presence of malachite green in Löwenstein-Jensen medium but, after a few days, the organisms were capable of multiplication, even when inoculated in small numbers. The presence of the organisms was easily detected by discoloration of the medium and by subcultivation on nutrient agar plates. Further experiments, not reported here, have shown that some Form 2 strains, when inoculated in a vegetative or in a sporulating stage, only yielded visible growth on Löwenstein-Jensen medium + malachite green when the cultures were aerated (by loosening the screw-caps) for about 10 sec. at intervals of 1-2 days. However, even when the presence of these organisms was not detected by visible colony formation or by discoloration of the medium, subcultures on nutrient agar yielded numerous colonies. All of the cultures from which Form 2 organisms were obtained and all of the uninoculated control cultures in previously reported experiments (Csillag, 1961, 1962, 1963*b*) were intermittently aerated and subcultivated on nutrient agar. (2) Stimulation of growth of Form 2 mycobacteria by the presence of Form 1 mycobacteria was not observed. (3) *Bacillus* spores germinated and formed colonies on Löwenstein-Jensen medium + malachite green and caused discoloration of the medium of a colour different from that produced by Form 2 organisms. Thus, there is evidence from these findings that vegetative Form 2 mycobacteria or *Bacillus* spores grow on Löwenstein-Jensen medium to an extent that is easily detectable by subculture on nutrient agar, and that conditioning of the medium by growth of Form 1 mycobacteria is not likely to increase the ease with which Form 2 mycobacteria can be isolated. The use of uninoculated medium which is otherwise treated in the same manner as the medium used for Form 2 isolation is therefore established as a valid control.

Further evidence that Form 2 mycobacteria do not arise as air contaminants is provided by the slopes which were deliberately exposed to the air. The contaminants that grew on Löwenstein-Jensen medium and on nutrient agar slopes after exposure to air were not as a rule species of *Bacillus*. In all the experiments on the production of Form 2 mycobacteria previously reported and in other unreported experiments no organisms other than Form 2 mycobacteria have ever been isolated by subculture from the test Löwenstein-Jensen slopes to nutrient agar. The absence of true contaminants in these experiments is not remarkable since the aeration procedure was carried out in a previously sterilized cabinet and took only a few seconds, whereas in the experiment reported here, only about half of the slopes which were deliberately exposed to the air for as long as 17 hr were contaminated.

In previous publications evidence, independent of the uninoculated controls, was presented that Form 2 mycobacteria were not air contaminants. This evidence can be summarized as follows: (1) A Form 1 culture, inoculated with old organisms yielded Form 2 organisms, whereas the same Form 1 strain, stabilized by frequent rapid passage on Löwenstein-Jensen medium, did not yield Form 2 organisms in the same experiment (Csillag, 1963*b*). (2) The isolation of Form 2 colonies on nutrient agar could be predicted by the previous appearance, within the Löwenstein-Jensen medium test cultures, of their early phases of development, which did not resemble the vegetative form or spores of *Bacillus* (Csillag, 1961). (3) The bacillary morphology of the organisms and, to some extent the colonial morphology, of Form 2 strains at their first isolation were specific for the Form 1 strain from which they were derived (Csillag, 1961). Form 2 mycobacteria have many characteristics in common with *Bacillus*, but differ in the production of culturable cocci (*Mycococcus*, Krassilnikov, 1959; Csillag, 1962). A fuller account of the production of these cocci will be reported elsewhere.

I wish to thank Dr D. A. Mitchison for many helpful suggestions and encouragement and Mr M. Clarke for his skilled technical assistance.

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