PROBLEMS IN THERMOPHILY

1. Are Thermophiles Derived from Mesophilic Forms ?

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SUMMARY

Studies with a number of isolates of facultative thermophiles tentatively identified as strains of *B. subtilis* tend to confirm the belief that thermophilic bacteria are mutants of the common mesophilic forms. The high frequency of occurrence of thermophilic forms from mesophilic populations and the changes in the nature of the colonies at different temperatures of incubation seem to show that heat may have a mutagenic action. A probable explanation for the growth of these mesophilic strains at elevated temperatures is also given.

INTRODUCTION

Ever since their discovery by Miquel,[18, 19] the heat-loving or the thermophilic sporeforming bacteria have attracted the attention of both the biochemist and the bacteriologist inasmuch as they present a 'biochemical anomaly' by being able to multiply and live at high temperatures at which activities of life would appear as near impossible owing to the coagulability of several proteins which constitute a significant portion of any living cell. A number of early as well as subsequent investigators have endeavoured to gather a good deal of information on their ecology, general biology, physiology, biochemistry and other aspects of their life, but still the knowledge gained with regard to the factors contributing to their ability to live at high temperatures is far from convincing and wherever any plausible explanation has been hypothesised concerning this problem it has not been always supported by experimental evidences. However, some of the more recent investigations made on them have helped not only to dispel some misconcepts we had about them, but have succeeded in contributing some valuable information regarding many aspects of their life including their nutritional requirements and metabolic products.[1, 4, 8]

Besides their peculiar problem of thermophilic habit of life, these bacteria pose some of the most interesting and intriguing problems one among which is the question of deciding whether or not they constitute thermophilic variants of mesophilic forms. Quite a few of the earlier investiga-

tors[17, 22–25] have entertained the idea of their derivation from the parent mesophilic bacteria because of the close resemblances between the two forms, as also because of the reported success of Tsiklinsky in converting a mesophilic strain of *B. subtilis* into a thermophilic variant, but the later experiments conducted by Golikowa,[7] Casman and Rettger[5] as also the more recent one by Imsenecki and Solnzeva[12] have not resulted in furnishing any convincing evidence in support of this view. Despite the discouraging results obtained from experiments designed to derive thermophiles from the mesophilic strains by gradual adaptations, because of several other considerations including the strongly favoured argument, *viz.*, the possibility of thermophilic mutants arising from a mesophilic population, as also for want of any other better explanation for this biological phenomenon, the derivation of thermophilic forms (mutants) from mesophilic parents has been accepted as a possibility. Moreover, the evidence presented by Kluyver and Baars[15] in connection with the adaptation of *Delsulphovibrio desulphuricans* to either mesophilic or thermophilic way of life is an evidence in favour of this view. Also, the recently reported work on the successful and consistent isolation of thermophilic forms from mesophilic populations[1] of several strains of *B. subtilis* and *B. megaterium* is another evidence in this direction. The purpose of the present communication is to report the results obtained in this laboratory on this aspect of thermophily which we believe is yet another approach in establishing the mesophilic origin of the thermophilic forms.

Experimental

Sources of Isolates.—The thermophilic micro-organisms prolifering at such high temperatures (55° C. or more) would suggest to one that they are capable of existing only in regions where the temperatures are comparatively high as in the tropics. However, this is not the case for they have been isolated from snow and the Arctic regions,[6, 7, 22] where the temperature never reaches even the minimum growth temperature requirements of these organisms. So wide is their distribution in nature, that they have been isolated from ocean bottom cores taken 10 miles off shore and at depths of 2,880 and 4,320 feet.[2] We, however, isolated our strains from such routinely resorted to sources as water, soil, hot springs, sugar and spoiled canned foods by making use of glucose nutrient agar at an incubation temperature of 55° C. Since Jones[13] in a review has suggested soil as the source of thermophiles, we have examined a large number of soil samples collected from various parts of India and have invariably found these organisms present. Table I gives a list of isolates made in this laboratory with their sources and the temperatures at which they can proliferate.

TABLE I

Isolates	Source	Growth at °C. 55	37	25–27 (R.T.)
		Group 1		
ESl	.. Earthworm soil ..	+	−	−
ES3	.. Earthworm soil ..	+	−	−
GS	.. Bombay garden soil ..	+	−	−
Hay	.. Hay infusion ..	+	−	−
Auto*	.. Autoclave water ..	+	−	−
Mango	.. Canned mango pulp ..	+	−	−
Air4	.. Bombay air ..	+	−	−
L1	.. Lucknow soil ..	+	−	−
L6	.. Lucknow soil ..	+	−	−
N2	.. Nagbir soil ..	+	−	−
N4	.. Nagbir soil ..	+	−	−
II	.. Vajreshwari spring II ..	+	−	−
RHS	.. Vajreshwari spring ..	+	−	−
Centre	.. Vajreshwari spring ..	+	−	−
G1	.. Gelatin ..	+	−	−
G2	.. Gelatin ..	+	−	−
Ld4	.. Bangalore Lab. dust ..	+	−	−
A1	.. Agra soil ..	+	−	−
A2	.. Agra soil ..	+	−	−
A3b	.. Agra soil ..	+	−	−
A4b	.. Agra soil ..	+	−	−
A5b	.. Agra soil ..	+	−	−
C1	.. Calcutta soil ..	+	−	−
C2	.. Calcutta soil ..	+	−	−
Dmo1	.. Damoh soil ..	+	−	−
Nag2a	.. Nagpur soil ..	+	−	−
		Group 2		
Sug1	Commercial sugar sample	+	+	+
Sug2	.. Commercial sugar sample	+	+	+
Chap.	.. Home made bread ..	+	+	+
EI5A	.. Intestine of Earthworm	+	+	+
EI5B	.. Intestine of Earthworm	+	+	+
Air1	.. Bombay air ..	+	+	+
Air3	.. Bombay air ..	+	+	+
P3	.. Poona soil ..	+	+	+
P7	.. Poona soil ..	+	+	+
P8	.. Poona soil ..	+	+	+
L4	.. Lucknow soil ..	+	+	+
N1	.. Nagbir soil ..	+	+	+
N3	.. Nagbir soil ..	+	+	+
Ia	.. Vajreshwari spring I ..	+	+	+
Ib	.. Vajreshwari spring I ..	+	+	+
III	.. Vajreshwari spring III ..	+	+	+

TABLE I (*Contd.*)

Isolates	Source	Growth at °C. 55	37.	25–27 (R.T.)
	Group 2 (*Continued*)			
TS1	.. Vajreshwari spring ..	+	+	+
TS2	.. Vajreshwari spring ..	+	+	+
Ld2	.. Bangalore Lab. dust ..	+	+	+
Ld5	.. Bangalore Lab. dust ..	+	+	+
2†	.. Bombay air ..	+	+	+
Am1	.. Amroati soil ..	+	+	+
Am2	.. Amroati soil ..	+	+	+
Nag1a	.. Nagpur soil ..	+	+	+
Nag1b	.. Nagpur soil ..	+	+	+
Nag1c	.. Nagpur soil ..	+	+	+
Nag2b	.. Nagpur soil ..	+	+	+
Nag2c	.. Nagpur soil ..	+	+	+
	Group 3			
ES2	.. Earthworm soil ..	+	±	−
EI8	.. Intestine of Earthworm	+	±	−
P4	.. Poona soil ..	+	(48 hours)	−
P5	.. Poona soil ..	+	+ (48 hrs.) +	−
P6	.. Poona soil ..	+	+	−
L2	.. Lucknow soil ..	+	+	−
A5a	.. Agra soil ..	+	(7 days) +	−
B1	.. Bina soil ..	+	+	−
B2	.. Bina soil ..	+	+	−
C3a	.. Calcutta soil ..	+	(48 hours) +	−
C3b	.. Calcutta soil ..	+	(3 days) +	−
C3c	.. Calcutta soil ..	+	(48 hours) +	−
C3d	.. Calcutta soil .	+	(48 hours) +	−
Nain	.. Nainpur soil ..	+	+	−
	Group 4			
A3a	.. Agra soil ..	−	−	−
A4a	.. Agra soil ..	−	−	−
K1	.. Katni soil ..	−	−	−
S1	.. Saugor soil ..	−	−	−
S2	.. Saugor soil ..	−	−	−

* Isolated from water which had undergone autoclaving several times.

† Could not grow at 55° C. when first isolated but could be trained to grow.

From the above table it is clear that the various isolates fall into the four groups on the basis of temperature requirements, *viz.*, (1) those that can grow only at 55° C. or obligate thermophiles; (2) those that can grow at all temperatures tested or facultative thermophiles; (3) those that fail to grow only at the lowermost temperature tested, and (4) those which grew initially at 55° C. but failed to grow at that or lower temperatures thereafterwards.

MATERIALS, METHODS AND RESULTS

For reasons that would become obvious, the experimental work presented in this paper was carried out exclusively with the group 2 isolates mentioned above. All the isolates made use of in this work, on preliminary examination, appeared to suggest that they are various strains of *B. subtilis* species.

It has been the consistent observation of others[1, 12] as well as our own that it is essential to prepare heavy suspensions for inoculations when working with thermophiles. A heavy suspension prepared in sterile distilled water with a 24-hour old growth was therefore utilised throughout this study. Although a 2 mm. loopful of this heavy suspension invariably gave a crowded growth on the plates incubated at 37° C. (the minimum temperature of growth for facultative thermophiles) it was absolutely essential to have this heavy inoculum, as otherwise no growth whatsoever could be obtained on the glucose agar plates incubated at 55° C.

It was consistently observed that when a suspension of any of the isolates in our collection which can grow at 37° C. was plated on glucose agar and incubated in duplicate sets for 24 hours at 37° C. and 55° C., there was invariably a heavy growth at the lower temperature of incubation whereas the incubation at the higher point resulted in the growth of only a few colonies. It occurred to us that it might be interesting to see what would happen if any plate showing a few colonies at 55° C. was incubated subsequently (after growth had ceased at 55° C.) for another 24 hours at 37° C. and when this experiment was followed with several isolates, it was indeed a surprise to note that the subsequent incubation at the lower temperature resulted in a heavy growth of cells which failed to grow initially at 55° C. It may be remembered here that all the isolates were initially obtained at 55° C. These results suggested that it would be worthwhile also to observe what changes, if any, would take place when a plate initially incubated at 37° C. was subsequently incubated for another 24-hour period at 55° C. Our observations on these experiments seem to suggest that the colony character undergoes a distinct change on subsequent incubation at the enhanced temperature as the detailed results recorded below indicate.

Results with isolate P3.—Only 9 colonies (see Fig. 1) of this strain developed when one loopful of the heavy suspension was plated out on glucose agar and incubated at 55° C. for 24 hours. With the same amount of inoculum, the plates incubated at 37° C, gave rise to approximately 250 colonies (see Fig. 2) during the same period of incubation. The results of one typical experiment are illustrated in the accompanying Plate. It may be observed from the illustrations that the colonies grown at 55° C. look smooth, round and moist in contrast to those grown at 37° C. which distinctly appear to be rough, wrinkled and dry. Now when the 55° C. growth was further incubated at 37° C. for another 24-hour period, more colonies developed which were typical of growth that would appear at 37° C. and it is very significant to observe that even these nine colonies developed at the initial incubation of 55° C. roughened and dried out almost in sympathy with the rest and tended to present a peculiar form distinguishable from the rest (see Fig. 3). On the other hand, when the growth secured initially at 37° C. was further incubated at 55° C., all of the dry colonies flatten and spread out by surrounding themselves with a glassy, mucilaginous mass which appeared to bring into a confluence all the colonial growth now under, perhaps, adverse conditions of temperature (see Fig. 4).

Results with isolate Nag2c.—Unlike the previous isolate, with this isolate equal number of colonies developed at the two incubation temperatures, but the colonies at 37° C. were large, irregular, wrinkled with knob-like projections on the margin in contrast to the smaller, smooth, round and regular colonies obtained at 55° C. When the plate originally incubated at 37° C. was kept at 55° C. for another 24 hours the colonies were surrounded by a slimy zone similar to that observed in the case of isolate P3, whereas the 55° C. growth when incubated at 37° C. for another 24 hours showed a change in the nature of the colonies in that they resembled those which appeared initially on the plate incubated at 37° C.

Other isolates.—Isolates Sug1, Sug2, N1, N3 behave in the same manner as isolate P3 except that there was no change in the nature of the colonies when the growth at 37° C. was subsequently incubated at 55° C. Isolates Ld2 and Ld5 resemble isolate Nag2c in that equal number of colonies develop at the two temperatures of incubation, but differ from it in the absence of any change in the nature of the growth when the 37° C. growth is subsequently incubated at 55° C. Finally, isolates Ib, III, 2 and EI5A give results identical with isolate P3.

DISCUSSION

The experimental evidence presented here is in favour of the mutagenic action of heat. In the case of isolate P3 the appearance of only 9 colonies at 55° C. in comparison to the 250 odd colonies developing at an initial incubation of 37° C. as well as on subsequent incubation of the 9 colony growth of 55° C. at 37° C. seems to suggest that only 9 living cells out of a population of 250 odd numbers are capable of growth under thermophilic conditions. This would mean that 9 heat-loving forms have arisen out of 250 odd mesophilic members, thus bringing the occurrence of thermophiles to 1 : 25, a proportion which is high as compared to what has been reported in the literature. Allen,[1] for instance, has shown a frequency of one thermophile for every million population of a strain of *B. circulans* and this was the highest frequency she had observed. Many investigations in the past have shown that the mutation rate is accelerated by an increase in the temperature. Muller[20] has reported an increase in gene mutation in *Drosophila* with increase in temperature. Plough[21] states that temperature only affects the rate at which mutants occur but does not have a mutagenic action. Low temperatures too have been reported to bring about an increase in the mutation rate.[3, 14] In fact it is widely accepted that heat may bring about an acceleration in the rate of mutation, but opinion seems to hold that it will not cause it. However, our observations seem to suggest such an action, or can the rate of mutation be accelerated to such an extent that a frequency of 1 : 25 be obtained ? Even Allen[1] seems to believe that a mutagenic action of heat is possible when she says " Until such time as a mutagenic action of heat is clearly demonstrated, it appears preferable to consider the outcome of the experiments as the selection of spontaneously occurring mutants, rather than the production of resistant individuals by the action of heat " (p. 139).

Further evidence in this direction of mutagenic action of heat is derived from the observation of Hansen[10] working with *Saccharomyces carlsbergensis*. This yeast when grown at 27° C. produced normal cells and typical colonies but when grown at 7° C. it produced elongated cells and colonies which differed considerably from those obtained at 27° C. He attributed this mutation to temperature. Lieske[16] tried to explain the wide occurrence of thermophiles by stating that these organisms arose by selection of the thermophilic variants of the mesophiles or as mutants due to the mutagenic action of heat. He was able to isolate thermophilic variants of actinomycetes from soil after it had been treated to destroy all thermophilic spores of actinomycetes, but failed to isolate thermophilic variants from pure cultures of mesophilic actinomycetes.

It may be pointed out here that one factor which has been suggested responsible for the growth of micro-organisms at elevated temperatures and which has not received sufficient attention is their ability to secrete a protective coating round the cell and Hampil[9] suggests this as the factor which prevents the coagulation of albumin in the cell either by removing moisture from the cell or by preventing moisture from passing into the cell through the cell wall. Hückel[11] could actually obtain by filtration from old cultures of *Pseudomonas æruginosa*, *E. coli* and staphylococci a non-specific substance secreted by the cells into the medium which he found could increase the heat tolerance of less resistant organisms. Our observations in the study of several isolates grown at 55° C. indicate a protective action of such a slimy coating. With these isolates not only did we observe under the microscope individual cells surrounded by a slimy substance but also well marked differences in the nature and character of colonies obtained at 37° C. and 55° C., the latter growth being characterised by a confluence of the colonies due to the presence of a slimy mucilaginous substance (see figures).

Acknowledgement

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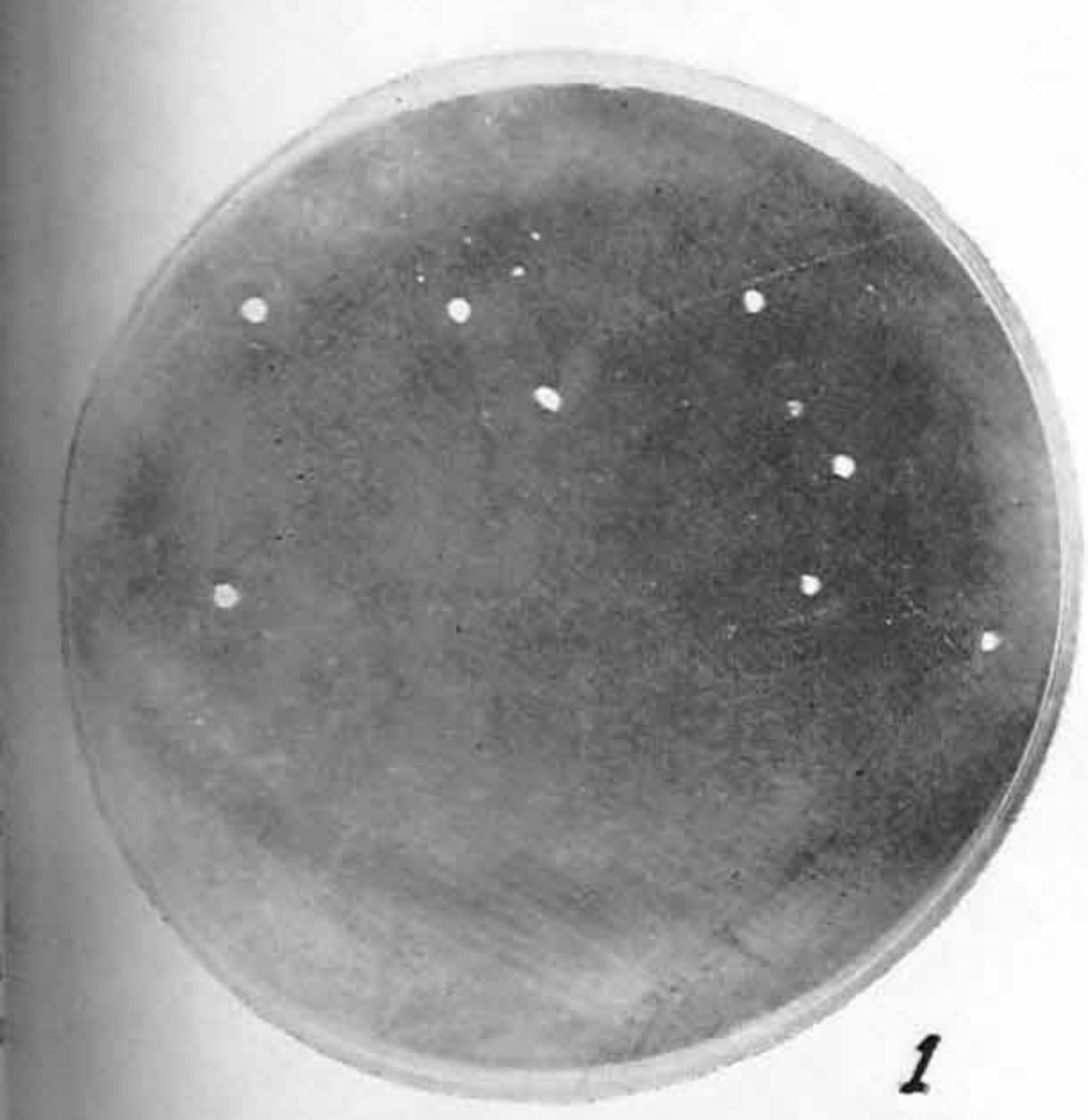
EXPLANATION OF PHOTOGRAPHS

FIG. 1. A 24-hour growth of P3 on 2% glucose agar at 55° C. Three-fourths natural size. Note the smooth growth and the small number of colonies.

FIG. 2. A 24-hour growth of P3 on 2% glucose agar at 37° C. Three-fourths natural size. Note the heavy growth of dry colonies.

FIG. 3. Results of incubating growth illustrated in Fig. 1 for another 24-hour period at 37° C. Note the peculiar circular growths in positions wherein the initial growth of the 9 colonies at 55° C. were located.

FIG. 4. Results of incubating growth illustrated in Fig. 2 for a further period of 24 hours at 55° C. Note the glassy, mucilaginous spreading mass around the colonies.



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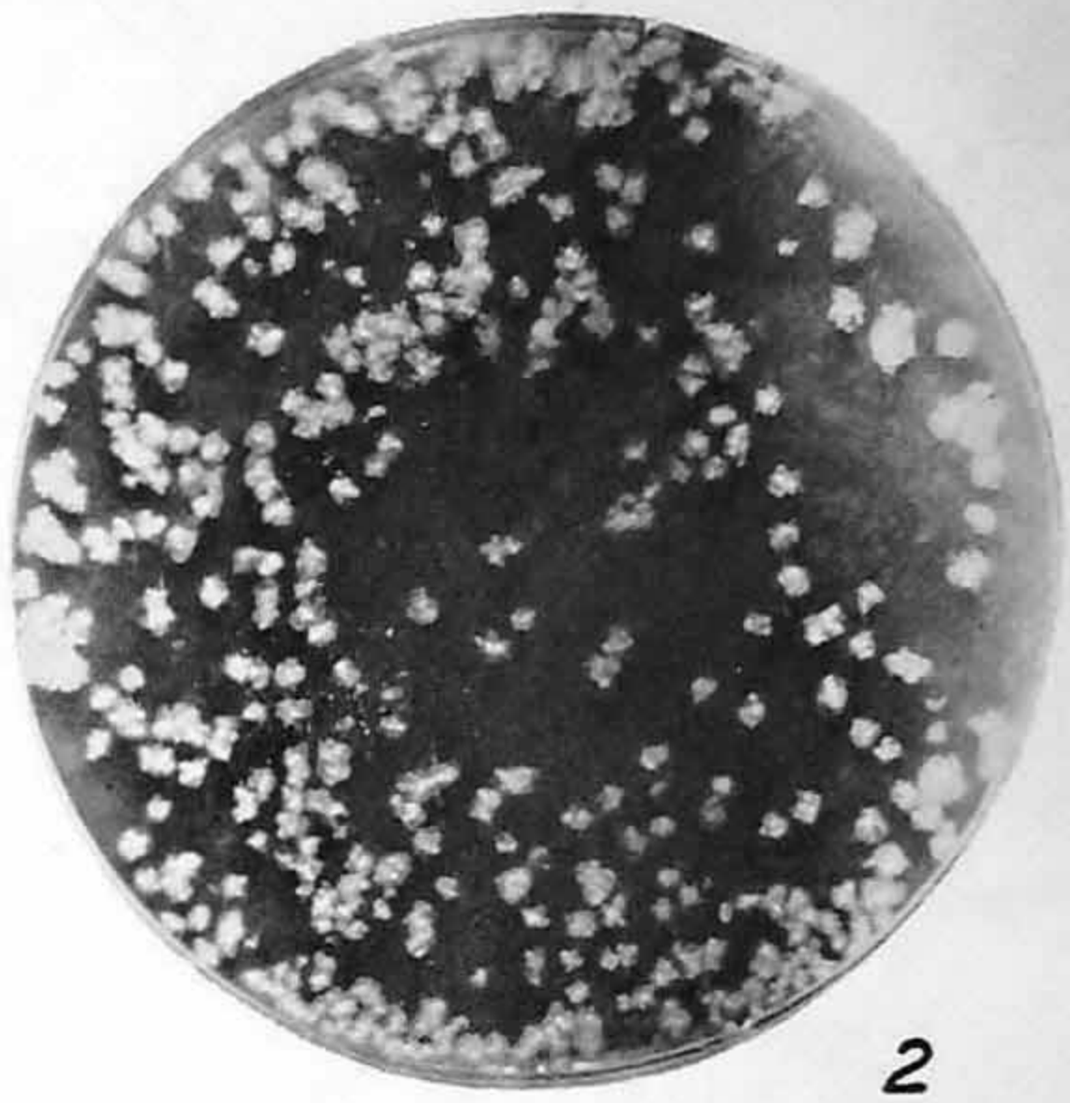

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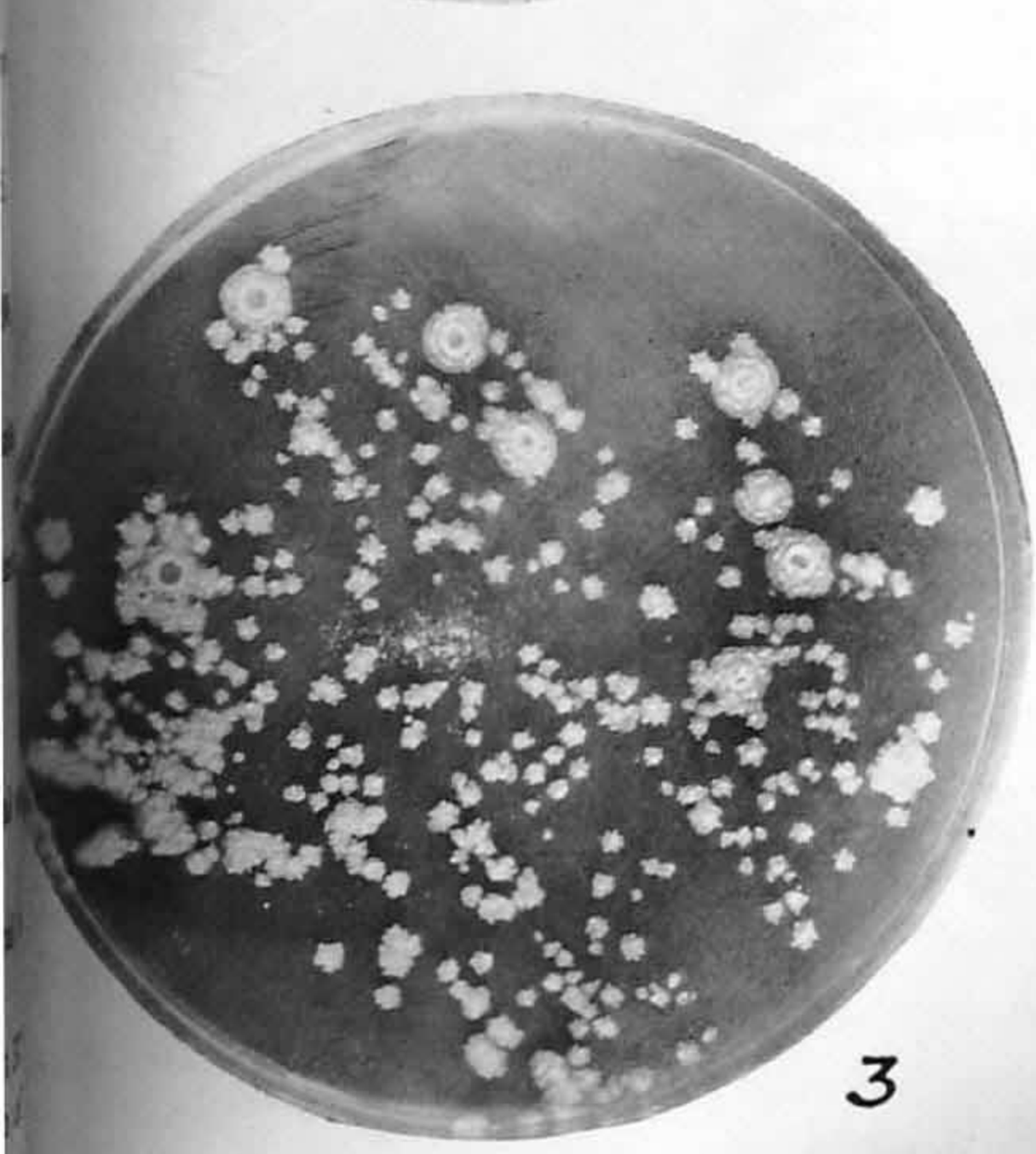

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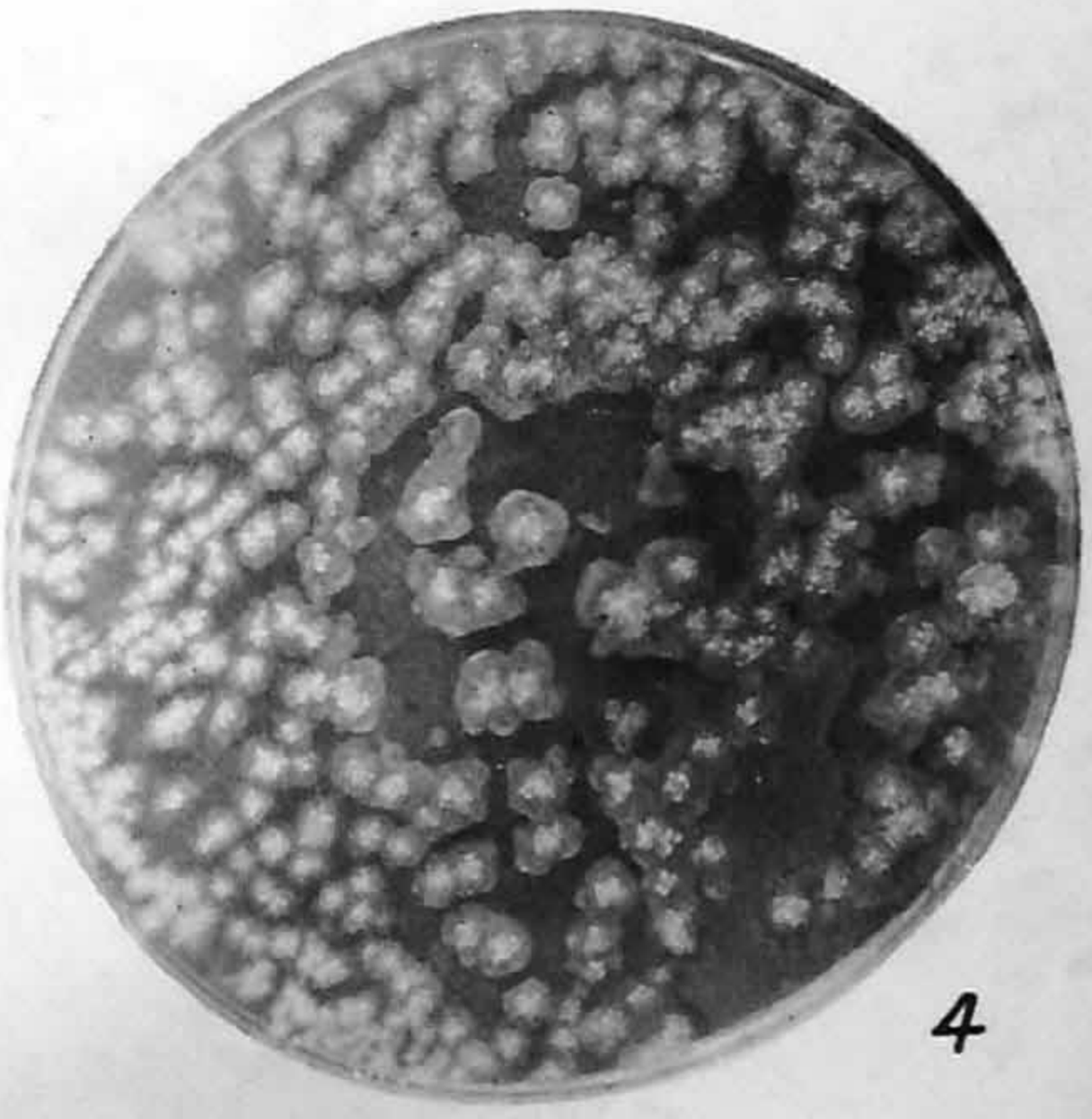

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