STALING IN BACTERIAL CULTURES

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Received for publication July 30, 1952

When a strain of bacteria is streaked on a nutrient agar or gelatin medium containing a large number of its own viable cells, no growth appears even after long incubation. Eijkman (1904) and Powers and Levine (1937) suggested that this phenomenon of "staling" was due to specific, autoinhibitory substances and that it might serve as a basis for selective and differential techniques in determinative bacteriology. Frankel and Wynne (1951) studied the inhibitory effect of staled media as an antagonistic reaction among different species of bacteria and interpreted the staling as an effect of direct or contact antagonism. The data reported here emphasize that this type of staling is a manifestation of the stationary growth phase and seems not to be caused by specific inhibitory substances or proximity of cells but rather probably by the exhaustion of some factor or factors essential to the anabolic processes of the cell. Previous studies on broth cultures (e.g., Barnes, 1931; Jordan and Jacobs, 1944) have drawn similar conclusions as to the nature of the factors limiting the total extent of growth in bacterial cultures.

METHODS

Cultures of the test organisms were grown for 48 hours at 37 C in 300 ml Erlenmeyer flasks containing 100 ml of 1.0 per cent N-Z case (Sheffield) in distilled water. The pH of the medium was 7.2 initially, 7.4 to 7.5 after growth.

Staled agar media were prepared essentially by the method of Powers and Levine (1937). The broth cultures were mixed with an equal portion of a 1 per cent N-Z case, 3 per cent agar medium which had been liquefied and cooled to 45 C. The surfaces of staled agar plates were streaked with 24 hour broth cultures of the test organisms. Growth after 48 hours at 37 C was compared with that on control plates prepared by mixing equal portions of sterile solutions of 3 per cent agar and of 1 per cent N-Z case. Longer incubation did not affect the results.

Potentiometric determinations of the degree of reduction in staled media were made with the Beckman model G pH meter. The tip of the platinum electrode was pressed firmly against the agar surface; the tip of the saturated calomel reference electrode was inserted into a drop of saturated KCl solution placed on the surface of the staled agar.

RESULTS AND DISCUSSION

From a staling spectrum obtained by streaking the test organisms on staled agar media which were prepared from each of the strains it was found that the autoinhibition on staled plates is not specific (table 1); although an organism seems always to be inhibited on its own staled agar, the inhibition by that medium may extend to other strains and species. Also, there is a relationship between the staling power of an organism and its ability to grow on the staled media of other species. For example, agar staled by *Bacillus cereus* inhibited all the other species tested, and *B. cereus* was able to grow on agar staled by any of the others, while *Micrococcus pyogenes* var. *aureus* had no effect on the other organisms and grew on none of the staled media.

These results indicate that the autoinhibition noted in staled agar (Powers and Levine, 1937; Coblentz and Levine, 1947) and the antagonism against other organisms reported to occur with staled agar media of *Gaffkya tetragena* and species of *Aerobacter* (Frankel and Wynne, 1951; Bowling and Wynne, 1951) are the same phenomenon. Our data from other experiments confirm the observations of the latter workers and of Barnes (1931) that autoinhibitory substances are not present in the cultures. No inhibitory effect could be demonstrated in cellular extracts or in filtrates from cultures 1 to 40 days old even after they had been concentrated by vacuum distillation at low temperatures. We also found that the extent of inhibition by the staled agar of a strain is corre-

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lated directly with the number of viable homologous cells present. As few as 12 million viable cells of *Escherichia coli* per ml of staled agar completely inhibited growth of the homologous organisms streaked on the surface.

Frankel and Wynne (1951) suggested that staling is the result of "direct antagonism" involving "close contact of living cells (of the staling strain) with cells of the inhibited species". However, we found that when staled agar was overlaid with a small amount of fresh agar and an interval of a few hours was allowed before this surface was streaked with susceptible strains, staling was manifested as usual. The staling organisms had not grown into the overlying medium. Frankel and Wynne observed no inhibition when they employed a similar technique, but they streaked the surface of the fresh layer immediately after the plates were prepared. If a very thin barrier of fresh agar is used, staling also will take place when the sensitive strain is inoculated immediately.

TABLE 1

*Growth of test organisms on staled agar media**

TEST STRAINS	GROWTH ON AGAR STALED WITH:													
	B. cereus, S721	*B. mycoides*, (S)	*B. mycoides*, (R)	*A. aerogenes*, UW	*A. aerogenes*, M	*A. aerogenes*, PU	*A. aerogenes*, P	*E. coli*, Sq	*E. coli*, H52	*E. coli*, M	*E. coli*, Gr	*E. coli*, 61	*E. coli*, 61A	*M. aureus*, 196
Bacillus cereus, strain S721	0	0	3	3	3	3	3	3	3	3	3	3	3	3
B. mycoides, strain (S)†	0	0	3	3	3	3	3	3	3	3	3	3	3	3
B. mycoides, strain (R)	0	0	0	1	0	0	1	3	3	3	3	3	3	3
Aerobacter aerogenes, strain UW	0	0	3	1	1	1	3	3	2	3	3	3	3	3
A. aerogenes, strain M	0	0	3	0	0	0	1	2	2	2	2	3	3	3
A. aerogenes, strain PU	0	0	2	0	0	0	1	3	2	2	2	3	3	3
A. aerogenes, strain P	0	0	2	0	0	0	0	2	1	1	1	3	3	3
Escherichia coli, strain Sq	0	0	2	0	0	0	1	0	0	0	0	2	2	3
E. coli, strain H52	0	0	1	0	0	0	1	0	0	0	0	2	2	3
E. coli, strain M	0	0	1	0	0	0	1	0	0	0	0	1	1	3
E. coli, strain Gr	0	0	1	0	0	0	0	0	0	0	0	1	1	3
E. coli, strain 61	0	0	1	0	0	0	0	0	0	0	0	0	0	3
E. coli, strain 61A	0	0	0	0	0	0	1	0	0	0	0	0	0	3
Micrococcus pyogenes var. *aureus*, strain 196	0	0	0	0	0	0	0	0	0	0	0	0	0	0

* Staled agar media prepared from 48 hour (37 C) cultures.

† *Bacillus cereus* var. *mycoides* (S): a nonrhizoid mutant of *Bacillus cereus* var. *mycoides* (R) with characteristics of *Bacillus cereus*.

0 = no growth, 1 = slight growth, 2 = moderate growth, 3 = good growth, equivalent to growth on control plates of fresh agar medium.

That the "inhibitory" state established in staled agar corresponds to the stationary phase of growth was substantiated by the following observations. When prepared in the manner described, staled agar media became slightly more turbid during aerobic incubation; on top and below the agar surface very small colonies not visible to the unaided eye were detected microscopically.

This increase in turbidity did not occur in areas of the plates which were covered with sterile cover glasses. Also, no growth was apparent after staled plates were incubated under strictly anaerobic conditions (alkaline pyrogallol method). On the other hand, surface growth that could be easily seen developed when staled agar plates of *E. coli* and *Aerobacter aerogenes* were incubated in vessels in which removal of oxygen was incomplete, according to a methylene blue indicator. It seems probable that the staled agar plates contained a maximum population for anaerobic conditions and that when a small amount of oxygen was present cells on the surface were able to grow while growth of cells in the deeper part of the medium was inhibited by the lack of oxygen due to its slow diffusion through the agar. These data suggest that in a staled agar medium the cells continue to reproduce until there is reached a viable population which is characteristic of the organisms under the environmental conditions. Other strains streaked on the surface of a staled medium would respond similarly.

Considerable evidence has been accumulated in support of the proposal that the maximum population attainable in a bacterial culture is regulated by the concentration and type of nutrients and the availability of oxygen (Barnes, 1931; Cleary, Beard, and Clifton, 1935; Jordan and Jacobs, 1944; Dagley, Dawes, and Morrison, 1951). It is interesting to note that although staling occurred as usual when a large number of cells was resuspended in fresh nutrient agar, abundant surface growth of the homologous organism was obtained often on staled agar to which was added 0.5 per cent or more glucose with enough phosphate buffer (usually 0.5 per cent) to maintain a pH near neutrality. Phosphate alone did not relieve the staled condition. Whether glucose was effective here as a more readily available energy source or carbon source for assimilation (Siegel and Clifton, 1950; Dagley, Dawes, and Morrison, 1951) should be considered.

The reducing power of a culture seems to be linked in some way to the factors controlling the maximum amount of growth. It was observed that the extent of reduction of 2,3,5-triphenyltetrazolium chloride (0.01 or 0.0025 per cent) in fresh and in staled media correlated with the staling power of the organisms, or conversely with their sensitivity to staling. Likewise, as determined potentiometrically the extent of reduction of staled media by the organisms correlated with staling ability (figure 1, *cf* table 1). This correlation was found also to hold true for strains within a species. The possible relationship of the reducing activity of a bacterial species to its ability to utilize more fully the nutrients available in a medium suggests a basis for further study of the fundamental problem of determining the factors controlling growth in bacterial populations.

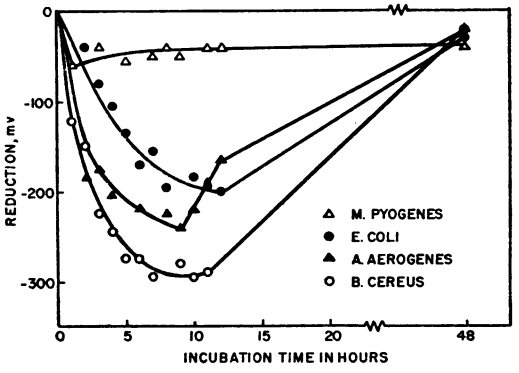

Figure 1. Relative reducing activity of various organisms in staled agar media. Reduction, mv = difference in millivolts between the potentiometric readings for staled agar and for uninoculated agar. Although absolute Eh values of control and staled agar varied in individual experiments, results were shown to be reproducible when expressed in terms of the difference between control and staled agar. Potentiometric readings (determined at 30 C for control agar) varied in different experiments from +10 to +170 mv at 0 hour and from −20 to +120 mv at 6 hours.

SUMMARY

From a comparative study of staling with strains of *Escherichia coli*, *Aerobacter aerogenes*, *Bacillus cereus*, and *Micrococcus pyogenes* var. *aureus* it was concluded that the autoinhibitory effect of staled agar is not specific. A relationship was demonstrated between the staling power of any strain and its sensitivity to staling by other strains and between the staling power and reducing ability of an organism.

No autoinhibitory substances were found in cultures of the test organisms. Although there is a definite relation between degree of inhibition and number of viable cells in the staled agar, the inhibition is not a result of "direct antagonism" by these cells. The staling effect in agar appears to be analogous to the cessation of growth in

broth cultures. Supply of nutrients and oxygen tension in relation to the reducing capacity of the organism seem to be implicated as controlling factors in this process.

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