SECONDARY COLONY FORMATION BY *BACILLUS SUBTILIS* ON EOSINE METHYLENE BLUE AGAR

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Discrete secondary outgrowths on colonies of bacteria growing on suitable indicator media constitute an often observed phenomenon of bacterial variation. Such outgrowths are produced by *Bacillus subtilis* and several other aerobic, sporeforming bacilli on eosine methylene blue agar. In the instance of *B. subtilis*, the cells constituting the secondary colony do not differ from the cells of the primary colony in their fermentative ability. The difference lies in the rate at which the acidity initially produced by fermentation is neutralized, resulting in the development of alkalinity in the medium. In previous studies (Lederberg, 1951; Ryan, 1952; 1955) of secondary colony formation in *Escherichia coli*, it has been shown that secondary colonies originate from mutants that arise in the primary colony and which are thereafter selected. The observations recorded in this study on secondary colony formation in *B. subtilis* are consistent with a similar explanation.

MATERIALS AND METHODS

Strains. Unless otherwise stated, a strain of *B. subtilis* var. *niger* (strain SAa) originally isolated from a sample of commercial sugar (Bhat and Iyer, 1955) was used in all experiments. The other strains of sporeforming bacilli used in some of the experiments were identified strains available in the laboratory.

Medium. Composition of the eosine methylene blue agar medium (EMB agar), used in all the experiments, was as follows: peptone, 1.0 g; NaCl, 0.5 g; meat extract (Oxo Laboratories), 0.3 g; glucose, 1.0 g; eosine, 0.04 g; methylene blue, 0.0065 g; agar, 2.5 g; glass distilled water, 100 ml. The glucose was sterilized separately and added to the rest of the sterile medium before pouring. Preliminary experiments in which the

meat extract was omitted from the above medium or where a defined medium (Demain, 1958) was used, indicated that such media were unsuitable in eliciting adequate development of secondary colonies.

RESULTS

Generality of the phenomenon. When a spore suspension of strain SAa was spread on EMB agar to yield from 10^2 to 10^4 colonies, some of the dark colonies that were produced gave rise, on further incubation, to secondary, paler outgrowths. The outgrowths were in the form of discrete secondary colonies confined to the area of the primary colony. They were of variable size and occurred anywhere on the primary colony from near its center to its periphery (Fig. 1a, b). They were not always well separated on the colony surface and occasionally, some marginally located ones spread out beyond the area of the primary colony. Of 15 strains of *B. subtilis* tested, all produced secondary colonies, although to a variable degree. Strains of *B. cereus*, *B. megaterium*, *B. circulans*, and *B. sphaericus* also produced secondary colonies under similar conditions.

Development of secondary colonies in relation to the number of primary colonies and the incubation period. The results of 4 experiments on the effect of incubation period on the number and size of primary and secondary colonies, are presented in Table 1. Secondary colonies were as a rule found to develop only after appreciable development of primary colonies. The number of secondary colonies generally increased with the incubation period reaching a maximum in 3 to 4 days on plates containing discrete primary colonies and in 5 to 6 days on plates containing a large number of confluent primary colonies. Examination of the results presented in Table 1 also show that with an increase in the number of primary colonies (or number of cells spread on the agar surface), there is an increase in the

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Fig. 1 (a and b). Secondary colonies of strain SAa on EMB agar ($\times 2.5$).

number of secondary colonies per plate. This increase is, however, not proportional to the number of primary colonies. For example, with an increase in the number of primary colonies from about 100 in experiment 2, to 150 in experiment 3 (Table 1), there is a disproportionately great increase in the number of secondary colonies per plate (about four times). On the other hand, on plates containing about 450 primary colonies (experiment 4), the number of secondary colonies that arise is lower than to be expected on a proportionate basis.

These experiments demonstrated the influence of the number of viable cells spread on a plate, but a question arose whether secondary colony formation was also influenced by the number of primary colonies; that is, by the manner of organization of the viable cells on the plate. To determine this, equal amounts of a thin spore suspension were spread in triplicate on three sets of plates of EMB agar and also on similar sets of identical media without EMB. The plates were incubated at 37 C and after an interval of 2 hr, the surfaces of one triplicate set of plates of EMB agar and another of the medium without EMB were respread. The second set of the two media was respread after 5 hr and the third set left unspread as the control. All plates, both unspread and respread, were further incubated for a period of 48 hr. After this period, besides counts of secondary colonies on EMB agar, the number of viable cells on the EMB-free plates was determined by washing off the growth from each plate with physiological saline and deter-

mining the number of cells in such washings by plate counts on nutrient agar. As may be observed from Table 2, resreading did not result in any significant alteration in the number of viable cells on the plate, whereas the number of secondary colonies on respread EMB agar plates was significantly greater. Taken together, the two groups of experiments (Tables 1 and 2) suggest that both the number of viable cells on a medium surface as well as their manner of distribution influence secondary colony formation.

Influence of area of inoculated medium surface. Further support for the above conclusion was derived from experiments in which a constant number of viable cells was spread on varying surface areas of the medium. The results of 3 experiments are presented in Table 3. It was observed that for a population size, where the inoculum permitted the development of discrete primary colonies (100 to 200 on each plate), an increase in the inoculated surface area was accompanied initially by an increase in the number of secondary colonies. Further increase in the surface area resulted in a decline in the number of secondary colonies (experiment 2). When the inoculum was so heavy as to result in confluent growth, the initial increase in the number of secondary colonies with increase in surface area was again evident but detection of the subsequent decline was not experimentally feasible (experiment 3). Secondary colony formation is thus a function of the number of primary colonies relative to the surface area available for growth.

TABLE 1

Effect of incubation period at 37 C on number and size of primary and secondary colonies (average of triplicates)

Expt No.	Incubation Period	Avg No. of Primary Colonies	Avg Diameter of Primary Colonies	Avg No. of Secondary Colonies per Plate
	days		mm	
I	1	20.0	4.0	0.0
	2	20.0	8.1	2.0
	3	20.0	9.7	2.0
	4	20.0	10.0	2.0
	10	20.0	10.0	2.0
II	1	95.0	3.5	0.0
	2	111.0	5.4	18.0
	3	115.0	7.2	18.0
	4	115.0	6.8	14.0
	5	115.0	6.5	12.0
	6	110.0	6.0	10.0
	7	110.0	6.0	10.0
	9	110.0	6.0	9.0
	10	110.0	6.0	9.0
III	1	143.0	3.8	1.0
	2	149.0	5.3	18.0
	3	149.5	6.5	61.5
	4	149.0	6.3	48.5
	5	151.0	6.2	47.5
	6	147.5	6.2	47.5
	7	145.0	6.2	46.5
	9	144.0	6.2	39.0
	10	144.0	6.2	39.0
IV	1	464.5	3.4	2.0
	2	483.0	4.7	78.0
	3	460.0	5.0	84.5
	4	460.0	5.0	78.0
	5	455.0	4.9	78.0
	6	455.0	4.9	73.5
	7	455.0	4.8	73.5
	9	455.0	4.8	57.5
	10	455.0	4.8	57.5
	1	45.5 × 10 ²	No discrete colony	3.5
	2	(calculated)		100.5
	3			110.5
	4			117.0
	5			122.0
	6			134.0
	7			134.0
	9			126.0
	10			113.0

TABLE 1.—(Continued)

Expt No.	Incubation Period	Avg No. of Primary Colonies	Avg Diameter of Primary Colonies	Avg No. of Secondary Colonies per Plate
	days		mm	
	1	4.5 × 10 ⁴	No discrete colony	98.0
	2	(calculated)		288.0
	3			309.0
	4			320.0
	5			380.0
	6			390.0
	7			380.0
	9			345.0
	10			325.0
	1	4.5 × 10 ⁶	No discrete colony	40.0
	2	(calculated)		84.0
	3			84.0
	4			96.0
	5			113.0
	6			140.0
	7			130.0
	10			130.0

The observed decrease in number of secondary and primary colonies on long incubation was more apparent than real. It was due to the growing colonies merging with each other.

Influence of depth of medium. By varying the depth of the medium and maintaining a constant area of the inoculated surface, it was sought to determine whether the volume of the medium or amount of nutrient available to the developing colonies could influence secondary colony formation. In these experiments, amounts of EMB agar in the range of 10 to 50 ml were poured into flat 9-cm petri dishes. Two dilutions of the spore suspension were then plated out separately on the media to give discrete and confluent growth, respectively. The growth of secondary colonies was recorded after an incubation period of 4 days by which time maximal development had occurred. The results of 4 experiments (Table 4) show that an increase in the depth of the medium is accompanied by an increase in the number of secondary colonies, the average size of the colonies remaining constant.

Influence of concentration of glucose in medium. The number of secondary colonies produced on EMB agar in the presence of increasing glucose concentrations up to 5.0% was studied using different concentrations of cells in the inoculum

TABLE 2
Effect of respreading primary colonies on the number of secondary colonies

	Plates Not Respread	Plates Respread after	
		2 hr	5 hr
Number of secondary colonies (average of 3 plates) on EMB agar	48.66	108.5	325.0
Number of viable cells (average of 3 plates) on each plate of EMB-free agar*	2.6×10^9	2.4×10^9	2.1×10^9

* Viable counts were made on suspensions washed from plates of EMB-free agar treated in an identical manner to each EMB agar plate.

TABLE 3
Effect of area of inoculated surface of medium on number of secondary colonies formed

Expt No.	Avg No. of Viable Cells per ml of Suspension	No. of Secondary Colonies Produced on Each of Following Inoculated Surface Areas (Diameter in cm)											
		2.0	2.5	3.0	3.5	4.0	4.5	5.0	6.0	7.0	8.0	9.0	10.0
I	0.9×10^3	42	52		70		100	122					
II	2.9×10^3	65		104		140		300			260	240	218
III	1.4×10^6	25		45		50		94	105	121	175	230	240

(Table 5). With increasing concentrations of glucose, there is an increase in the average size of primary colonies, in the number of primary colonies giving rise to secondary colonies, and in the average number of secondary colonies per plate (Fig. 2a to f). Secondary colonies do arise in small numbers even in the absence of glucose though under these conditions, the development of both primary and secondary colonies is poor. Since glucose affects the growth of primary colonies, increased secondary colony formation must be considered as a probable result of the interaction between the glucose and the growing primary colony.

Differences between cells from primary and secondary colonies. Morphological and cytological observations on cells from primary and secondary colonies revealed no apparent differences. Physiologically, the cells ferment glucose rapidly with acid production. In preliminary experiments, it was observed that cells from the region of the secondary colony when inoculated into peptone water containing glucose and an indicator, ferment the sugar as effectively as cells from the rest of the colony. However, on further incubation of the tubes it was observed that after the development of acidity as judged by the indicator, the reaction gradually became alkaline. This

TABLE 4
Influence of depth of medium on number of secondary colonies

Expt No.	Avg No. of Cells Spread on Each Plate	No. of Secondary Colonies Produced on Surface of Petri Dishes (Diameter 9.0 cm) Containing Varying Amounts of EMB Agar (ml)				
		10	20	30	40	50
I	1.0×10^6	3	34	225	300	400
	1.0×10^2	15	20	25	25	78
II	8.0×10^6	0	90	200	400	500
	8.0×10^3	52	83	170	265	354
III	1.5×10^5	5	49	100	371	
	1.5×10^2	4	83	170	265	354
IV	1.4×10^6	0	95	300	450	550
	1.4×10^4	80	250		400	
	1.4×10^2	15		16	25	78

Note: No appreciable differences in the size of secondary colonies were observed.

reversal to an alkaline pH occurred more rapidly in tubes inoculated from the secondary colony area. The observation was confirmed for several independently arising primary and secondary

colonies. By serial transfer and selection of cells from the primary and secondary colony area, it was possible to isolate after approximately 60 generations a primary colony type and two

secondary colony types, none of which produced secondary colonies on further subculture. The physiological difference observed between cells from the primary and secondary colony area, persisted in these isolates. This finding demonstrated that the cells from the secondary colony were genetically distinct from those comprising the rest of the primary colony.

In further experiments, 250-ml flasks containing 75 ml of sterile nutrient broth and 1% glucose, were inoculated separately with cell suspensions of the isolated primary and secondary colony types. Changes in the pH of the media were subsequently followed at regular intervals. It can be observed (Fig. 3) that while the initial fall in pH is similar for both primary and secondary colony types, cells from the latter reverse the pH of the medium to an alkaline level earlier than cells from the former.

Prevention of secondary colony formation. In an attempt to prevent the development of secondary colonies, the EMB agar was buffered by the addition of 1.0% calcium carbonate. Secondary colonies were rare or absent on media containing calcium carbonate even on prolonged incubation (up to 15 days) whereas control plates without

TABLE 5
Effect of varying concentrations of glucose on secondary colony formation

Expt No.	Concn of Glucose	Avg No. of Primary Colonies with Secondary Colonies	Avg No. of Primary Colonies without Secondary Colonies	Avg No. of Secondary Colonies per Plate
I	<i>g/100 ml</i>			
	0.0	15.0	91.0	17.0
	0.1	30.0	62.0	100.0
	0.5	85.0	0.0	300.0
	1.0	94.0	0.0	350.0
	3.0	97.0	0.0	400.0
	5.0	96.0	0.0	500.0
II	0.0	Sheet of primary growth—no discrete colonies		54.0
	0.1			86.0
	0.5			110.0
	1.0			220.0
	3.0			250.0
	3.5			300.0

Fig. 2 (a-f). Influence of glucose concentration on secondary colony formation by strain SAa on EMB agar ($\times 0.45$): (a) 0.0%, (b) 0.1%, (c) 0.5%, (d) 1.0%, (e) 3.0%, (f) 5.0%.

Fig. 3. Developmental pH in nutrient broth + 1.0% glucose inoculated with isolated primary colony and two secondary colony strains.

Fig. 4 (a and b). The ability of calcium carbonate to inhibit secondary colony formation by strain SAa on EMB agar ($\times 0.75$): (a) with calcium carbonate, (b) without calcium carbonate.

the buffer developed secondary colonies in 2 to 3 days (Fig. 4a, b).

DISCUSSION

The physiological difference between cells constituting the secondary colony and cells constituting the rest of the primary colony lies in the ability of the former to reverse the acidity pro-

duced in a peptone medium more rapidly than the latter. It may be reasoned that the highly proteolytic nature of cells of *B. subtilis* enabled them to attack the peptone in the medium rapidly and convert them to amino acids. Simultaneously, fermentation of the glucose in the medium renders the environment acidic. In such an acidic environment, the amino acids will be attacked pref-

erentially by decarboxylases to yield alkaline amines which reverse the acidity. Such a reversal occurs more rapidly with secondary colony cells apparently because they are endowed with greater decarboxylase activity. The validity of this reasoning is supported by the experiment in which secondary colony formation is prevented by simply incorporating an adequate and readily available buffer in the medium. The inference is that the presence of the buffer ensures against the development of acidity in the environment and thus indirectly against selection of the cells that have greater decarboxylase activity and which eventually form the secondary colony.

Consideration of the various factors found to influence secondary colony formation suggests that these are consistent with a mutation-selection hypothesis although the question of whether the mutant cells which form the secondary colony are selected by the environment or are induced by the selective conditions, is not determined experimentally. The formation of secondary colonies, in this particular instance, is found to be influenced by various complex interacting factors among which must be considered the age and size of the primary colony, the number of cells and their arrangement into colonies on the surface of the medium. A reasonable inference is that as a prerequisite to secondary colony formation, (i) the primary colony must attain a population size sufficiently high to raise the probability of a mutation to 1.0, (ii) the cells constituting the primary colony must alter the growth environment by the depletion of nutrients or accumulation of acidic metabolic products, and (iii) such an altered environment must be specially capable for the selective outgrowth of mutant cells. This selective outgrowth constitutes the secondary colony. The production of a mutant cell potentially capable of forming a secondary colony does not necessarily assure the appearance of a discrete visible secondary colony, for phenotypic expression is subject to various interacting factors both within the confines of the primary colony and in the medium as a whole. The increase in secondary colonies produced on a plate with the number of cells plated is to be expected, whereas the lack of proportionality in increase must be attributed to barriers against phenotypic expression which also operate when the surface area or depth of the medium is altered. Spreading a developing primary colony increases the number

of colonies as this serves to separate mutant cells after they have arisen. The relationship between the concentration of glucose and the degree of secondary colony formation is understandable as an increase in glucose concentration would lead to the rapid production of an acidic and therefore favorable environment for the mutant cells. Prevention of the development of such an acidic environment by means of a buffer added to the medium is an effective way of inhibiting the development and appearance of secondary colonies. The fact that the cells constituting the secondary colony can be separated away from the primary colony cells and that such separated cells continue to show their physiological difference on serial subculture, establishes that they are genetically distinct.

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SUMMARY

Colonies of sporeforming bacilli growing on eosine methylene blue agar produce discrete secondary outgrowths. With a strain of *Bacillus subtilis* var. *niger* these secondary colonies originate from mutant cells that arise in the primary colony. It is postulated that growth of the primary colony involves the simultaneous production of acids from the glucose and amino acids from the peptone in the medium, followed by decarboxylation of the amino acids to alkaline amines that result in a reversal in hydrogen ion concentration. Cells constituting the secondary colony can be separated and shown to bring about a more rapid reversal in pH as compared to cells present in the rest of the primary colony. Furthermore, this distinctive property is retained on subculture. Observations on various factors influencing secondary colony formation, although consistent with a mutation-selection hypothesis, suggest that the expression of mutant cells is subject to various interacting factors within the colony and in the environment on the plate.

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