THE EFFECT OF EOSINE AND METHYLENE BLUE ON STREPTOMYCIN-DEPENDENT *BACILLUS SUBTILIS*

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Introduction

Recently *Shah et al* (1960) demonstrate that colonies of spore forming bacilli grown on eosine methylene blue agar produce discrete secondary outgrowth. This distinctive property was retained on subculture. It seems that eosine and methylene blue has some mutagenic effect on those microorganisms. But based on their experiment they suggested that the expression of mutant cells were subjected to various interacting factors, within the colony and in the environment on the plate.

In our laboratory when streptomycin-dependent strain of *Bacillus subtilis* ATCC 558 was grown on the eosine methylene blue media, it was found that not only the formation of secondary colony was effected but the change of cell morphology and cell content was observed. It appears that the methylene blue alone has a strong mutagenic effect on the st-d strain of *B. subtilis*.

In this experiment wild type and streptomycin-resistant strain of *B. subtilis* was used as control and the effect of eosine and methylene blue on the formation of secondary colony, and the change of cell morphology and cell content of st-d strain of *B. subtilis* were studied.

Materials and Methods

Bacterial strains: Three types of *B. subtilis* ATCC 558 were used in this experiment. The wild type of *B. subtilis* ATCC 558 was never in contact with any antibiotics and was sensitive to streptomycin. Both the streptomycin-resistant type (st-r) and streptomycin-dependent type (st-d) were isolated from the wild type by *Chiu* (1964) one step pattern selection method.

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Medium: Composition of the eosine methylene blue basal medium used in this experiment was as follows: Difco nutrient broth, 8 gm; agar-agar, 25 gm; glucose 10 gm; distilled water, 1000 ml. Glucose was sterilized separately and then added to the rest of the sterile media. Four series of indicator media were prepared by adding prescribed quantities of eosine and methylene blue stock solution to basal medium.

(a) Eosine media, with various concentrations of eosine i. e. 800 μg/ml, 400 μg/ml, 320 μg/ml, 160 μg/ml, 80 μg/ml, 40 μg/ml, 20 μg/ml, 0 μg/ml.

(b) Methylene blue media, with various concentrations of methylene blue i. e. 130 μg/ml, 65 μg/ml, 52 μg/ml, 26 μg/ml, 13 μg/ml, 6.5 μg/ml, 3.25 μg/ml, and 0 μg/ml.

(c) MB-E media, with constant concentration of methylene blue (65 μg/ml) and various concentrations of eosine as in (a).

(d) E-MB media, with constant concentration of eosine (400 μg/ml) and various concentrations of methylene blue as in (b).

Test tubes each containing 20 ml of the above mentioned media were sterilized in autoclave, then poured into sterile petri dishes to prepare media plates. For cultivation of st-r type and st-d type, streptomycin was added to the above mentioned indicator media to a concentration of 1000 μg/ml. The pH of the media was preadjusted at 7.6 before sterilization to counterablance the lowering of pH because of the acid nature of streptomycin. Preparation of streptomycin plates was carried out as follows: Melted stock media (pH at 7.6) at temperature 50°C was poured into sterile petri dishes (9 cm in diameter) containing 0.1 ml of 200 mg/ml streptomycin sulfate solution and mixed thoroughly before the solidificating media. The composition of Stewart's G-medium was yeast extract, 2 gm; K_2HPO_4, 1 gm; $MnSO_4$, 0.1 gm; $MgSO_4$, 0.8 gm; $ZnSO_4$, 10 gm; $CuSO_4$, 10 gm; $CaCl_2$, 10 gm; $FeSO_4 \cdot 7H_2O$, 1 gm; Glucose, 4 gm; Difco nutrient broth 1000 ml; agar-agar, 20 gm; at pH 7.2. Preparation of bacterial spores: Cells were cultivated on Stewart's G-media for 4 days for the wild type without streptomycin and for both the st-r type and st-d type with streptomycin sulfate (6000 μg/ml). Clean spores were collected by Albertson's (1950) two-phase system, suspended in normal saline solution, and were kept at 65°C for 30 minutes in order to remove the vegetative cells. The spore suspensions were stocked in a freezer at-4°C ready for use.

Cultivation: One loop of the spore suspension was inoculated by the streak technique on each appropriated medium plate. The cultures were then incubated at 37°C for 10 days. The experiment was made in triplicate.

Chemicals: Dihydrostreptomycin sulfate (Takeda, Japan) was dissolved with sterile distilled water to a concentration of 200 mg/ml which was stored in a

freezer at temperature below-10°C. The eosine used in this study was eosine Y, purchased from Kanton company, Tokyo, Japan. The methylene blue used in this study was methylene blue N. F., purchased from the Coleman and Bell company, U. S. A.

Method of observation: At incubation intervals of 24 hr's, 48 hr's, 4 days, 7 days, and 10 days, the number, shape, size and color of colony, and the growth of secondary colony on the media were observed by naked eye at first, then five smears were made from a single secondary colony on each plate and were subjected to Gram stain (Hucker's method), simple stain (Loeffer's alkaline methylene blue stain), spore stain (Schaefferfulton modification of Wirtz method), cell wall stain (Rv Webb method), and fat stain (Burdan's method) respectively. The stainability and morphological change of the cells were examined microscopically.

Results

General aspect of colonies: About 100 to 200 colonies grew out from one loop of spore suspensions cultivated on various eosine and methylene blue agar plates. These colonies can be classified into two types. One type was hard rough form colony, with rough and wrinkled surface. This type showed little further growth and yielded few secondary colony. The other type was soft smooth form colony, with a little protrusion on the surface. After incubation for 3 to 4 days, secondary colonies of various sizes appeared along the periphery of the primary colonies of this type (Fig. 5). 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.

The growth of colonies after 24 hr's cultivation on various kinds of media: The growth on eosine media are shown in Fig. 1. the growth of wild type, st-r type and st-d type varied with eosine concentration. But the effect of eosine on growth was not very remarkable. Both wild type and st-r type developed a few secondary colonies after 4–10 day's cultivation. But no secondary colony was observed on st-d type, even after 10 days of cultivation. The effect of methylene blue on growth of wild type, st-r type was much more remarkable than that of eosine. As shown in Fig. 2. the growth dropped sharply with increase of methylene blue concentration, especially with the st-d type. No secondary colonies were formed on all these cultures, even after 10 days of cultivation. The growth on MB-E media are shown in Fig. 3. the inhibiting effect of MB-E on growth of st-d type was much more than that of the wild type and st-r type, The st-d type developed secondary colonies much better

than the wild type and st-r type, when eosine concentrations were higher above 80 μg/ml. The growth on E-MB media are shown in Fig. 4., both growth and spore germination of all those type were inhibited by the increase in the concentration of methylene blue. But the growth war better compared whth that on the methylene blue media. It is interesting to note that the rate of formation of secondary colonies of the st-d type on this media was faster than that of the wild type and st-r type.

Microscopic observations were done by means of simple stain, Gram stain, cell wall stain, spore stain and fat stain. After 24 hours cultivation on eosine media, no remarkable morphological changes for all three types were detected from microscopic observation. However, after an incubation period of 4 to 7 days, spore formation at high eosine concentration (400 μg/ml) was found. After 24 hour's cultivation on methylene blue media, no special morphological change for wild type and st-r type was found. There was no morphological change found for st-d type in the methylene blue concentration ranging from 3.25 μg/ml to 13.0 μg/ml after 24 hour's cultivation. However, cells became curved, filamentous and coarse, septa of cells became thicker and increased of fat granules, when the concentrations over 26 μg/ml (Fig. 10, Fig. 11). No spores were found nevertheless. After 24 hours cultivation on MB-E medsa

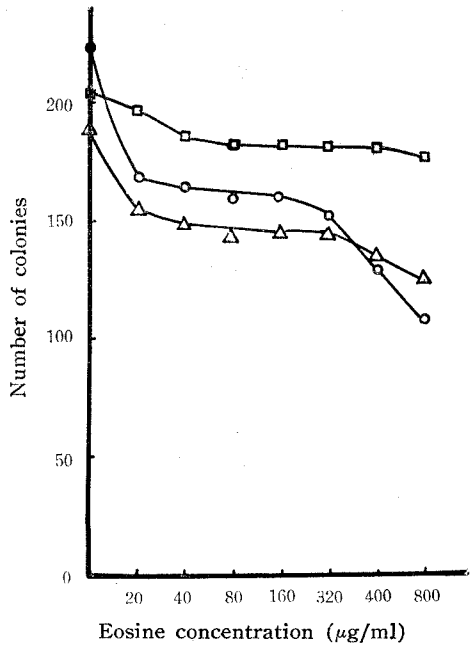


Fig. 1. Effect of eosine on growth and spore germination of *B. subtilis*

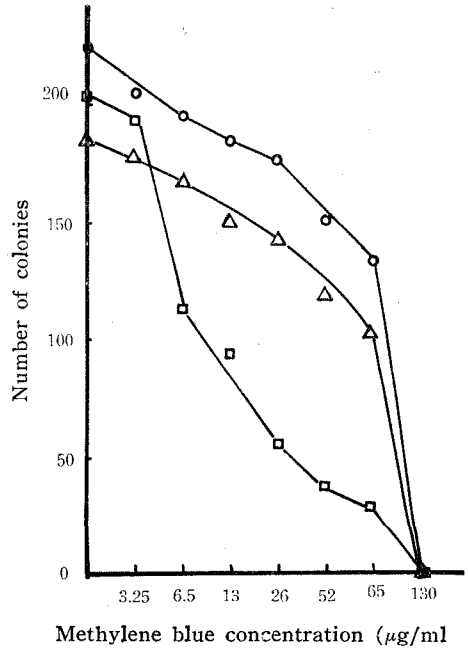


Fig. 2. Effect of methylene blue on growth and spore germination of *B. subtilis*

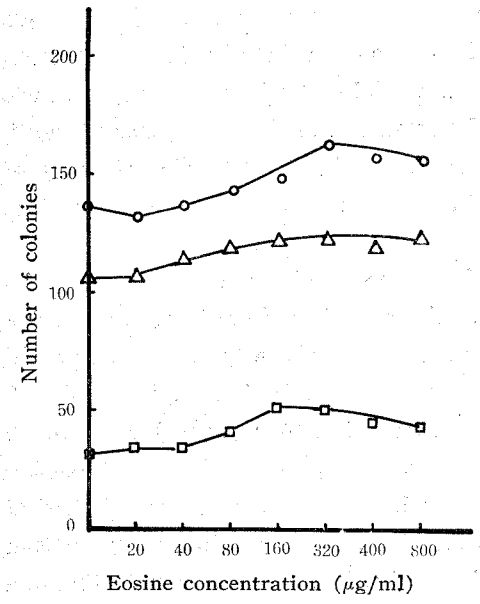


Fig. 3. Effect of MB-B on growth and spore germination of *B. subtilis*

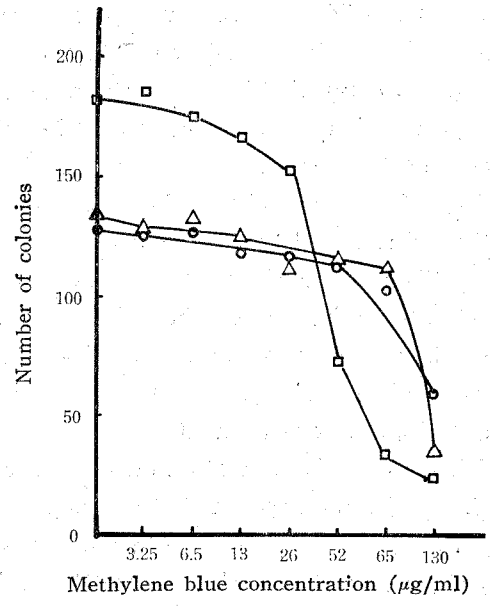


Fig. 4. Effect of E-MB on growth and spore germination of *B. subtilis*

Incubation period: at 37°C for 24-48 hours.
Judged by the colony number and size.
—○— Wild type.
—△— Streptomycin-resistant type.
—□— Srteptomycin-dependent type.

Explanation of the Plate Figures

Fig. 5. Secondary colonies of st-d type on EMB media following incubation of 7 days at 37°C. (×1)

Fig. 6. Wild type after 24 hours cultivation on EMB media Gram stain showing normal shape (× 2500)

Fig. 7. Wild type after 24 hours cultivation on EMB media cell wall stain showing normal shape (× 2600)

Fig. 8. Wild type after 24 hours culivation on EMB media fat stain showing no fat granule. (× 2500)

Fig. 9. St-d type after 7 days cultivation on eosine (400 μg/ml) media spore stain showing 70-80% spores and some cells became filamentous form (×2500)

Fig. 10. St-d type after 24 hours cultivation on methylene blue media (65 μg/ml) the size was enlarged as 5 to 10 times, and cells become irregular forms (×2500)

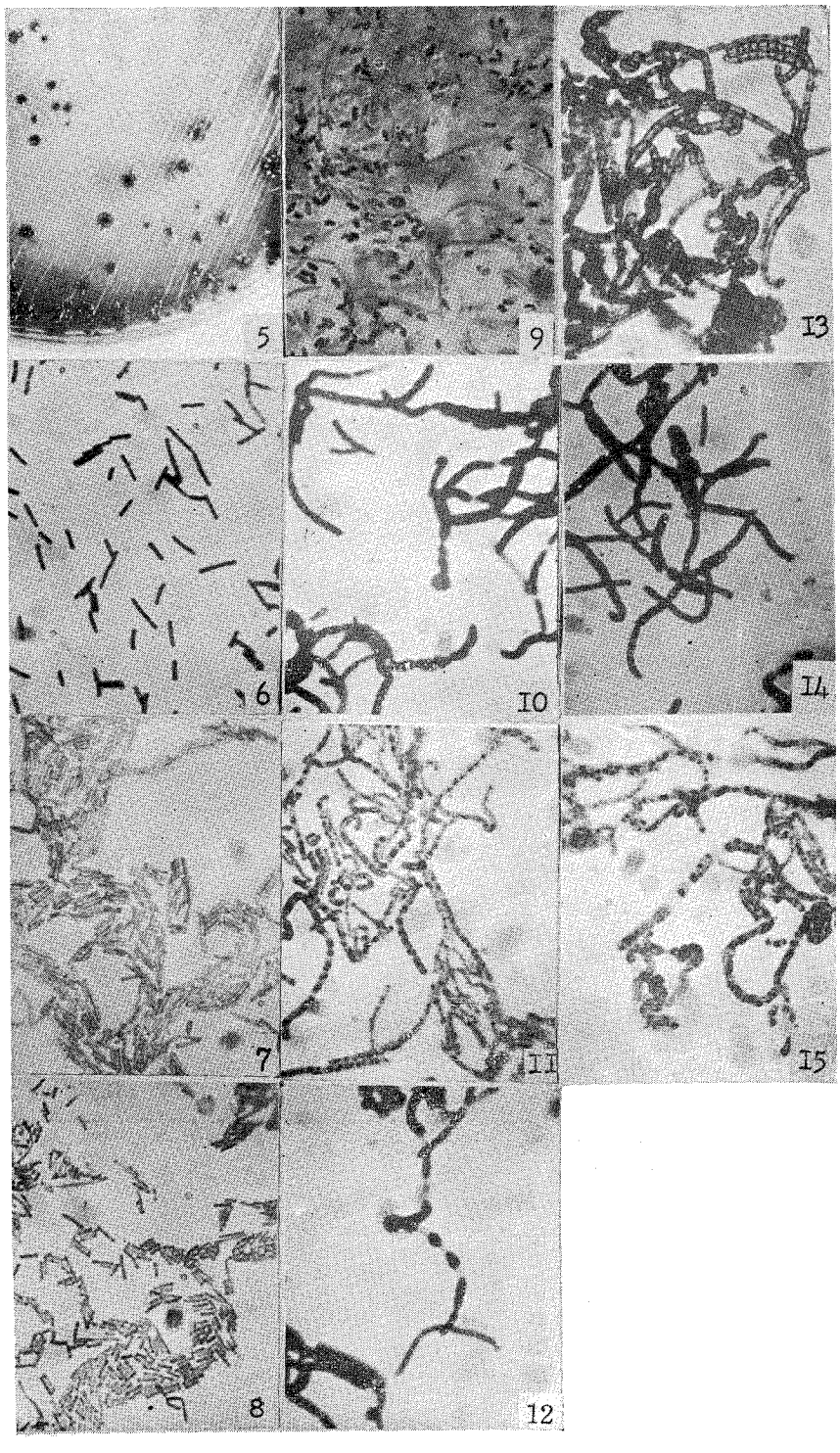
Fig. 11. St-d type on methylene blue media (52 μg/ml) by fat stain fat particles were noted. (× 2500)

Fig. 12. St-d type on eosine (40 μg/ml) and methylene blue (65 μg/ml) media Gram stain showing cells become irregular, short and curved shape. (× 2500)

Fig. 13. St-d type on eosine (20 μg/ml) and methylene blue (65 μg/ml) media cell wall stain showing septa become thicker.

Fig. 14. St-d type on eosine (400 μg/ml) and methylene blue (52 μg/ml) media Gram stain showing cells become slender, filamentous, surved and coarse shapes (×2500)

Fig. 15. St-d type on eosine (400 μg/ml) and methylene blue media (52 μg/ml) by fat stain 1-2 large fat particles found in irregular cells.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no remarkable morphological change for wild type and st-r type was found. But for the st-d type, the morphological change was greater at lower sosine concentrations. Irregular shapes, thicker cell wall and large fat granules were observed at eosine concentration below 80 μg/ml (Fig. 12, Fig. 13), whereas almost normal forms were found at 400 μg/ml concentration. No spores were found at eosine concentration below 400 μg/ml. After 24 hour's cultivation on E-MB media, no special morphological change for wild type and st-r type was found. There was no morphological change found for st-d type at methylene blue concentrations below 26 μg/ml. At methylene blue concentration over 26 μg/ml cells became irregular shapes and large fat granules were found (Fig. 15). Marked thickening of septa and roughenning of cell wall surface were observed. No spores were found at methylene blue concentration 130 μg/ml.

Discussion

Spore formation was observed at eosine concentrations over 320 μg/ml. To study the effect of eosine on spore formation of *B. subtilis*, the wild type, st-r type and st-d type were cultivated on G-media, nutrient agar and eosine (400 μg/ml) media for comparison. The result revealed that spore formation of the st-d type on eosine media was more luxuriant than that on G-media. As shown in Table 1. This suggests that eosine has an intimate relation with the spore formation of the st-d type.

Table 1.

Relation between spore formation and media

Incubation period	Spore formation % on:								
	Nutrient media			G-media			Eosine (400 μg/ml) media		
	W.	st-r	st-d	W.	st-r	st-d	W.	st-r	st-d
24 hr's	0	0	0	1-2	0	2-3	0	0	0
4 days	3-5	1-2	3-5	50-60	10-15	30-40	15-10	1-2	30-40
7 days	5-10	3-5	5-10	50-60	10-15	50-60	50-60	3-5	70-80

W.: Wild type of *B acillus subtilis* ATCC 558

st-r: Streptomycin-resistant type, isolated from wild type by the spontaneous mutation and selection method.

st-d: Streptomycin-dopendent type, isolated from wild type by the spontaneus mutation and selection method.

Incubation period: At 37°C for 24 hour's, 4 days and 7 days.

Both eosine and methylene blue inhibited the spore germination and the growth of *B. subtils*. To compare the inhibiting effect on growth with that of other dyes, the wild type, st-r type and st-d type were cultivated respectively on

the same basal media with various concentractions (from 0.1 μg/ml to 10 μg/ml) of basic-fuchsine, safranine or malachite green. The results were shown in Table 2. basic-fuchsine, safranine and malachite green at concentration less than 20 μg/ml had inhibiting effect on the growth of all the three types. On the other hand, even at a concentration of 800 μg/ml and 65 μg/ml of eosine and methylene blue respectilvely, the same inhibitory effect as that of the other three dyes was not reached. The organisms cultivated on the basic-fuchsin, safranine, malachite green and eosine media were noted to show no special morphological change by simple stain cell wall stain and fat stain. Whereas those on the methylene blue do. Emphasis can be made here about the increase of fat granules in cytoplasm and irregularity of cell wall. From this point of view, a question arises further study as how the methylene blue plays its role in the fat metabolism of st-d type of *B. subtilis*. Perhaps it is necessary that extensive investigations on the metabolic role of methylene blue in the st-d type of *B. subtilis* must be first carried out before these questions can be answered.

Table 2.

Effect of various dyes on growth of B. subtilis

Dyes / Strans / Conc. (μg/ml)	Basic-fuchsin			Safranine			Malachite green		
	W.	st-r	st-d	W.	st-r	st-d	W.	st-r	st-d
0.1	##	##	##	##	##	##	##	##	##
0.2	##	##	##	##	##	##	##	##	##
0.4	##	##	##	##	##	##	+	+	+
0.5	##	##	##	##	##	##	+	—	—
1	##	##	##	##	##	##	+	—	—
5	##	##	##	##	##	##	—	—	—
10	+	+	+	+	+	##	—	—	—
20	+	+	+	—	—	+	—	—	—
40	—	—	—	—	—	—	—	—	—
80	—	—	—	—	—	—	—	—	—
100	—	—	—	—	—	—	—	—	—

W.: Wild type of *Bhcillus subtilis* ATCC 558

st-r: Streptomycin-resistant type, isolated from wild type by the spontaneous mutation and selection method.

st-d: Streptomycin-dependent type isolated from wild type by the spontaneous mutation and selection method.

Incubation period: at 37°C for 24 to 48 hours.

—: No colonies

+: Few colonies

##: More colonies than +

##: Many colonies, same as the maximum growth in the control which has no dye

Summary

1. Both eosine and methylene blue had inhibiting effect on the growth and the spore germination of the wild type, streptomycin-resistant type and streptomycin-dependet type of *Bacillus subtilis* ATCC 558. The inhibiting effect of methylene blue was more remarkable than eosine. The combination with methylene blue, eosine reduced the inhibiting effect of methylene blue.
2. High concentration of eosine above 400 μg/ml had a favorable effect on spore formation of B. subtilis, especially for the streptomycin-dependent type.
3. Methylene blue caused irregular change of cells shape at concentration above 26 μg/ml. The effect of methylene blue was especially remarkable for streptomycin-dependent type.
4. Methylene blue caused increase of fat granules in cells and irregularity of cell wall structure for streptomycin-dependent type at concentration over 26 μg/ml.

Eosine 及 Methylene blue 對鏈黴素依存性枯草菌之作用的研究

吳 崇 洋

eosine 及 methylene blue 對野生種 (wild type)，鏈黴素耐性種 (streptomycin-resistant type) 及 鏈黴素依存性種 (streptomycin-dependent type) 枯草菌之芽胞發芽及生長均有阻止作用，二者之間 methylene blue 之作用較 eosine 爲顯著。但當二者混合，則 eosine 能使 methylene blue 的作用減低。

依存性枯草菌在 eosine 之濃度 400 μg/ml 以上時芽胞之形成較爲良好，於 methylene blue 之濃度在 26 μg/ml 以上時，能引起細胞形態不規則的變化，並促使脂肪顆粒的增加，但野生種及耐性種對 eosine 及 methylene blue，並不產生依存性種所生的現象。

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