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Ethidium Bromide-Resistant Mutant of *Bacillus subtilis*

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An ethidium bromide-resistant mutant (EB8) derived from a Marburg strain of *Bacillus subtilis* was found to be conditionally resistant to 10 μg of ethidium bromide per ml. Expression of resistance is complete only during vegetative growth at incubation temperatures above 30 C in complex medium or minimal medium supplemented with Casamino Acids. Strain EB8 is cross-resistant to acriflavine and proflavine. The ethidium bromide resistance marker is co-transduced with *hisA1* at a frequency of 6% and is located to the right of *hisA1* on the *B. subtilis* chromosome as it is usually represented on the map. Incorporation of [5-^3H] uridine by strain EB8 showed that ribonucleic acid synthesis in both whole cells and protoplasts is ethidium bromide-resistant.

Ethidium bromide (EB) (2,7-diamino-9-phenylphenanthridium-1-ethyl bromide) has been used for a variety of biological purposes. EB induces petite mutations in yeast (19), it has been used to eliminate penicillinase plasmids and resistance transfer factors from bacterial cells (2, 16), it is an inhibitor of nucleic acid biosynthesis (20), and it functions as a mutagen in bacterial cells (13).

EB binds to nucleic acids in vitro and is a commonly used intercalating agent (22). In addition, EB binds to cell membranes with a subsequent enhancement of fluorescence (6).

Resistant variants of bacteriophage QB have been reported (18), but few references in the literature describe bacterial mutants resistant to EB (7). Here we describe the characterization of one such resistant mutant with unique conditionally expressed resistance. The possible involvement of the cell membrane in this resistance is discussed.

MATERIALS AND METHODS

Bacterial strains. *Bacillus subtilis* strains used in this study are listed in Table 1.

Media. Media used were: antibiotic medium no. 3 (PAB, Difco); tryptose blood agar base (TBAB, Difco); AK agar (BBL); and Shaeffer's ×2 sporulation medium (9). SFL-2 medium of Landman and Forman (11), supplemented with 1% vitamin-free Casamino Acids (CA, Difco) and 1% glucose, was used for protoplast formation. The minimal medium used in this study was Spizizen's minimal (1) plus 0.2 or 0.5% glucose (MG), and in some experiments this medium was supplemented with CA. When used for growth of auxotrophic mutants, MG medium was supplemented with the required amino acids at 50 μg per ml. MG agar medium contained 15 g of Noble agar (Difco) per liter.

Mutant isolation. *B. subtilis* Marburg spores (1.2×10^8 viable spores/ml) were treated with ethyl methanesulfonate for 20 min as described by Haworth and Brown (9). Samples were diluted 2×10^{-2} with sterile distilled water and filtered on membrane filters (Type HA, 0.45 μm pore size, 86 mm diameter; Millipore). The filters were washed once with sterile distilled water, transferred to TBAB plates, and incubated for 2 h at 37 C to allow for expression of EB resistance. Then the filters were transferred to TBAB plates containing 10 μg of EB per ml (TBAB-EB10 plates), and incubated for 20 h at 37 C. EB-resistant colonies were picked and purified by several transfers on TBAB. One of these isolates (EB8) was chosen for this study.

Transduction. Transduction experiments using PBS1 phage were carried out as described by Haworth and Brown (9).

Multiplicities of infection ranged from 0.5 to 2 in these experiments, and all incubations were carried out at 37 C.

Direct selection of prototrophic transductants was done on MG (0.2% glucose) plates supplemented with the amino acid requirements (50 μg/ml) for nonselected auxotrophic markers. The prototrophic transductants were picked onto appropriately supplemented MG plates and incubated overnight. The "picked clones" were replica-plated onto TBAB, incubated for 6 h, and then transferred to TBAB-EB10 plates. After 18 to 20 h of incubation the TBAB-EB10 plates were scored for EB-resistant clones.

The *rou-1* marker was scored on AK agar plates after 36 h of incubation, at which time the rough

phenotype was easily discernible from the smooth phenotype (8).

The *uvr-1* marker was scored on TBAB medium supplemented with mitomycin C (0.05 μg/ml) (10).

Direct selection of EB-resistant GSY1057 transductants was accomplished by filtering the transduced cells on 0.4-μm Nuclepore membrane filters (General Electric), placing the filters on TBAB plates, and incubating these plates for 3.5 h to permit expression of EB resistance. The filters were then transferred to TBAB plates containing 5 μg of EB/ml (TBAB-EB5 plates) and incubated for 24 h. The EB-resistant colonies were transferred to TBAB-EB5 plates, incubated for 20 h, replica-plated to TBAB plates, incubated for 6 h, and transferred to MG (0.2% glucose) plates supplemented with methionine (50 μg/ml). After a 48-h incubation period, the plates were scored for histidine prototrophs.

Spore production. Spores were collected after 48 h of growth in ×2 Shaeffer medium with shaking at 37 C and purified by using the "Y" system of Sacks and Alderton (17).

Germination, outgrowth, and growth studies. All germination, outgrowth, and growth experiments were done at a rotary shaking speed of 250 rpm. Optical density measurements were made at 660 nm.

Protoplast preparation. A modified version of Clive and Landman's procedure was used for preparing protoplasts (4). MG (0.5% glucose) medium (25 ml), supplemented with 1% CA (MG + 1% CA), was inoculated from a streaked TBAB plate (incubated for 12 h at 37 C). After incubation with shaking for 16 h at 37 C, 0.1 ml of this culture was inoculated into MG + 1% CA (25 ml) and incubated for 6 h with shaking at 37 C. One milliliter from the 6-h culture was diluted with 9 ml of SFL-2 medium, supplemented with uridine (10 μg/ml), in each of three 250-ml flasks, and incubated with shaking for 90 min at 37 C. Lysozyme was added to a final concentration of 300 μg/ml, and the cells were incubated for an additional 30 min without shaking at 37 C. These cells were then centrifuged at 8,000 × *g* for 10 min, washed once with MG + 1% CA medium containing 0.67 M sucrose, and resuspended in 1 ml of the same medium. The resuspended cells were examined microscopically to ascertain that protoplast formation was complete.

Radioisotope experiments. Cells used in either whole cell or protoplast [5-^3H] uridine incorporation experiments were incubated as indicated above under protoplast preparation, except that in whole cell experiments cells were incubated with shaking for 120 min in 10 ml of MG + 1% CA (0.67 M sucrose) medium supplemented with uridine (10 μg/ml). These cells were washed and resuspended as described above under protoplast preparation. Each of two 125-ml flasks containing 10 ml of MG + 1% CA (0.67 M sucrose) medium supplemented with [5-^3H] uridine (0.2 μCi per 10 μg per ml) were inoculated with 0.2 ml of the resuspended cells. The two flasks were preincubated for 20 min to allow for equilibration of intracellular uridine pools. At zero-time, EB was added to a final concentration of 10 μg/ml. Duplicate 0.5-ml samples were taken at 10-min intervals and placed in tubes containing 0.5 ml of 10% trichloroacetic acid, followed by the addition of 2 ml of 5% trichloroacetic acid. The sample tubes were held on ice for 30 min or more, after which each sample was filtered on a glass fiber prefilter (25 mm diameter; Millipore) and washed once with 3 ml of 5% trichloroacetic acid and then twice with 95% ethanol. The filters were placed in scintillation vials, allowed to dry overnight at room temperature, and then counted after the addition of scintillation fluid (5 ml).

Fluorescence measurements. Relative fluorescence intensities of protoplasts suspended with EB were measured with an Aminco-Bowman spectrophotofluorometer at excitation and emission wavelengths of 542 and 592 nm, respectively. Concentrations of protoplasts were determined by optical densities at 620 nm.

RESULTS

Studies on growth, germination, and sporulation were carried out at various temperatures in several different media to characterize the effects of EB on wild-type and EB8 cells physiologically.

Cross-resistance to acriflavine and proflavine was also studied to see whether strain EB8 is cross-resistant to other chemicals known to bind to deoxyribonucleic acid by intercalation (12).

Growth of the Marburg and EB8 strains in the presence of EB. Growth of the Marburg strain in PAB medium is strongly inhibited by 2.5 μg of EB/ml and completely blocked by 10 μg/ml (Fig. 1A). But growth of strain EB8 in PAB (Fig. 1D) is only slightly inhibited by 2.5 μg of EB/ml, and even in the presence of 10

TABLE 1. *Strains of B. subtilis*

Strain	Genotype	Origin	Parent strain	Source
Marburg	Wild type			N. Sueoka
EB8	*ebr*	EMS[a] mutagenesis	Marburg	Isolation
Br19	*hisA1, trpC2, rou-1*		SB1	F. Young
OSB169	*cysB, his, trp, strA*		Marburg	L. Brown
GSY1057	*metB3, hisA1, uvr-1*			J. Hoch
GSY1058	*argC4, hisA1, uvr-1*			J. Hoch

[a] EMS, Ethyl methanesulfonate.

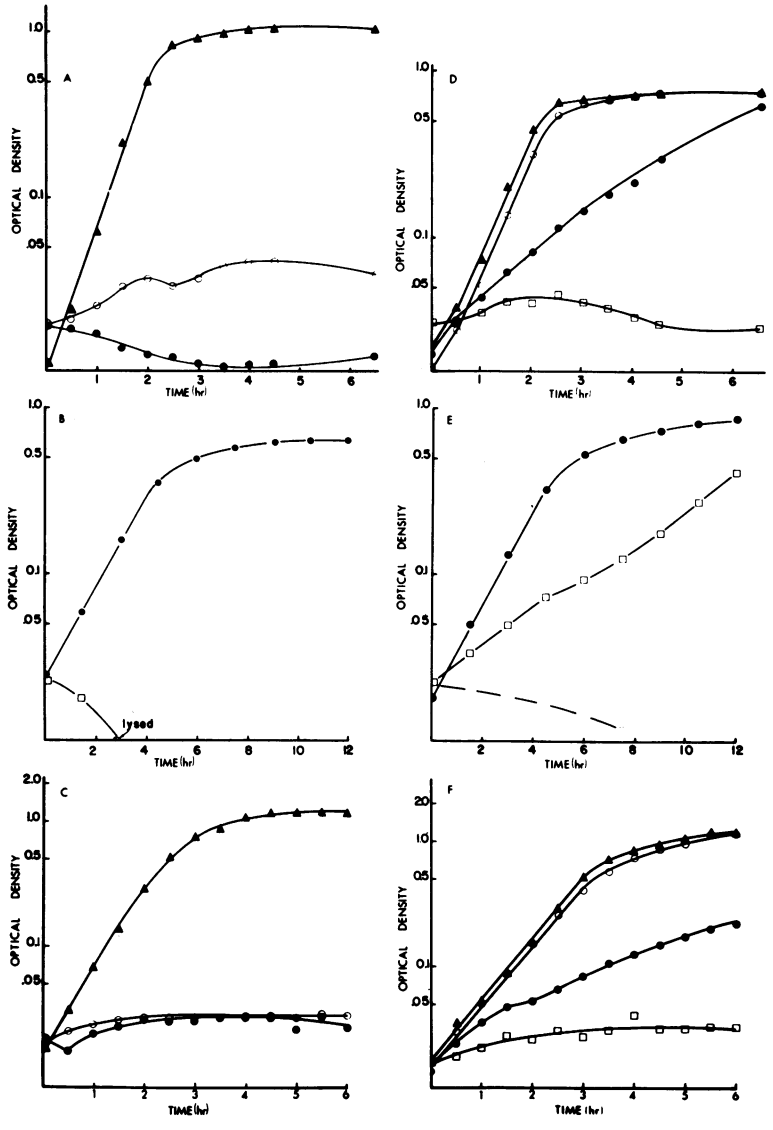

FIG. 1. *Growth of Marburg and EB8 at 37 C in the presence of EB. A, Marburg in PAB; D, EB8 in PAB. Symbols: ▲, ○, ●, and □ represent 0, 2.5, 10, and 20 μg/ml, respectively. B, Marburg in MG (0.5% glucose); E, EB8 in MG (0.5% glucose). Symbols: ●, □, and ○ represent 0, 2.5, and 10 μg of EB per ml, respectively. C, Marburg in MG (0.5% glucose) and Casamino Acids (0.2%); F, EB8 in MG (0.5% glucose) and Casamino Acids (0.2%). Symbols are as in A and D.*

μg/ml, growth remained logarithmic throughout the experiment. However, growth of strain EB8 was blocked by 20 μg of EB/ml. In Figure 1B it can be seen that 2.5 μg of EB/ml completely inhibited growth by the Marburg strain in MG medium. Figure 1E shows that growth of strain EB8 in MG, containing 2.5 μg of EB/ml, remained logarithmic, whereas it was completely blocked in the presence of 10 μg/ml. It was found, however, that this incomplete expression of resistance could be overcome by adding CA, as can be seen in Fig. 1C and F. From these results it appears that amino acids are essential to the full expression of resistance. However, testing of strain EB8 on MG plates containing different amino acid pools, as well as EB (10 μg/ml), failed to reveal any single amino acid that would permit full expression of resistance in minimal media.

Effect of temperature on the expression of

EB resistance by strain EB8. Strain EB8 grew at 37 and 47 C on TBAB medium containing 10 μg of EB/ml but not at 25 or 30 C. To see if this result could be repeated in liquid media, strain EB8 was incubated at 37 C in PAB containing EB (10 μg/ml) and then shifted to 30 C. After the shift to 30 C growth was strongly inhibited, and after 5.5 h long filamentous forms were observed microscopically.

Cross-resistance to acriflavine and proflavine. Table 2 summarizes the results from experiments on cross-resistance to acriflavine and proflavine. Strain EB8 grew on TBAB containing 5 and 10 μg of acriflavine per ml, whereas the Marburg strain did not. Strain EB8 was also cross-resistant to 10 μg of proflavine per ml when incubated at 30, 37, or 47 C, and to 20 μg/ml at 30 or 37 C, but resistance at this concentration was marginal at 47 C. Under these same incubation conditions and concentrations of proflavine, the Marburg strain failed to grow.

From the above results it is interesting to note that strain EB8 was cross-resistant to proflavine when incubated at 30 C, in contrast to the results obtained with EB.

Effect of EB on sporulation of EB8. Sporulation of strain EB8 was blocked when grown in the presence of EB (10 μg/ml) on either AK agar or in ×2 Shaeffer sporulation medium. After 16 h of growth at 37 C with vigorous shaking in ×2 Shaeffer medium containing 10 μg of EB/ml, microscope examination revealed no spores or intermediate stages. However, the cells were highly motile and their morphology appeared normal. In contrast, microscope examination of the control culture (without EB) showed many spores and intermediate stages after the same incubation period. Also, strain EB8 appeared to sporulate normally on AK agar at both 37 and 47 C.

Germination and outgrowth of strain EB8 in the presence of EB. Germination, as followed by a loss in refractility and drop in turbidity, did not appear to be affected by EB (10 μg/ml) when it was added to germinating spores of strains Marburg or EB8 at the onset of germination. But, outgrowth was blocked in strain EB8 except when EB was added after the first 45 min. Figure 2 shows that when EB is added at times later than 45 min, outgrowth takes place; however, microscope examination of the outgrowing cells showed that many curved and twisted forms were also present.

Chromosomal location of the EB resistance marker. Genetic mapping studies using PBS1 bacteriophage-mediated transduction were carried out since the chromosomal location of the EB resistance (*ebr*) marker might give some clue as to the mode of resistance. Results from

TABLE 2. *Cross-resistance of strain EB8 to acriflavine and proflavine*

Strain	Acriflavine concn (μg/ml)	Proflavine concn (μg/ml)	Incubation temp (C)	Growth[a]
Marburg	5		37	−
EB8	5		37	+
Marburg	10		37	−
EB8	10		37	+
Marburg		10	30	±
EB8		10	30	+
Marburg		20	30	−
EB8		20	30	+
Marburg		10	37	−
EB8		10	37	+
Marburg		20	37	−
EB8		20	37	+
Marburg		10	47	±
EB8		10	47	+
Marburg		20	47	±
EB8		20	47	±

[a] Growth was scored on TBAB medium containing the indicated concentrations of either acriflavine or proflavine. Symbols: −, no growth; +, growth; ±, marginal growth.

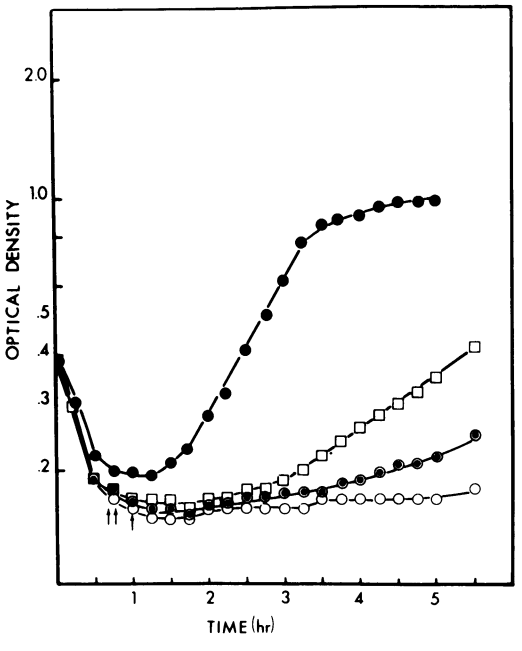

FIG. 2. *Germination and outgrowth of EB8 in PAB at 37 C in the presence of EB. Arrows indicate times of addition of EB (final concentration of 10 μg/ml). Symbols: ●, ○, ◉, and □ represent: no addition of EB, addition at 40 min, 45 min, and 50 min, respectively.*

two-factor crosses show that the *ebr* marker is co-transduced with the *hisA1* marker at a frequency of 6% but does not link with either the *cysB3* or *argC4* markers. This would imply that *ebr* is located to the right of *hisA1* as represented in Fig. 3, since *argC4* does not appear to be linked to *hisA1* (23), and *cysB3* co-transduced with *hisA1* at a frequency of 22% (5). Results from the analysis of three-factor crosses involving the *uvr-1* and *rou-1* markers (Table 3) confirm this location. Figure 3 shows the approximate location of the *ebr* marker in relation to some other markers in the *hisA1* region of the *B. subtilis* chromosome.

Effect of EB on RNA synthesis in whole cells and protoplasts. The location of the *ebr* marker in a region on the *B. subtilis* chromosome known to contain genes regulating cell wall synthesis (3, 23) suggested that resistance to EB might be due to a modification of the cell wall. This possibility was tested by removal of the cell wall (protoplast formation) from the Marburg and EB8 strains and then comparing the effect of EB on ribonucleic acid (RNA) synthesis in both the whole cells and protoplasts. RNA synthesis was measured by the uptake of [5-^3H] uridine into trichloroacetic acid-insoluble material, and Fig. 4A and B show that RNA synthesis, in both whole cells and protoplasts of the Marburg strain, was completely inhibited by EB (10 μg/ml). On the other hand, RNA synthesis in whole cells and protoplasts of strain EB8 is inhibited by 10 μg of EB/ml, but not blocked (Fig. 4C, D). Moreover, RNA synthesis in whole cells and protoplasts of strain EB8 appears to be inhibited to about the same degree. This suggests that the cell wall is not necessary for the resistance of RNA synthesis (and probably resistance of cell growth in general) to EB.

Fluorescence measurements on protoplasts of Marburg and EB8 suspended with EB. Since the experiments described above ruled out modification of the cell wall as being the major mode of resistance of strain EB8 to EB, we thought that protoplasts of the Marburg and EB8 strains might enhance EB fluorescence to a different degree. If this were true, then it might imply that cell membranes of the Marburg and EB8 strains bind EB differently, either qualitatively, quantitatively, or both (6). By using an excitation wavelength of 542 nm, we were unable to detect any significant difference in relative fluorescence intensities when different concentrations of protoplasts of the Marburg and EB8 strains were suspended in MG + 1% CA medium (containing 0.67 M sucrose) in the presence of 10 μg of EB/ml. When protoplast suspensions were diluted with distilled water, a large increase in the relative fluorescence intensity of EB (10 μg/ml) was seen with ruptured protoplasts of both the Marburg and EB8 strains, and again, no difference could be observed between the two strains.

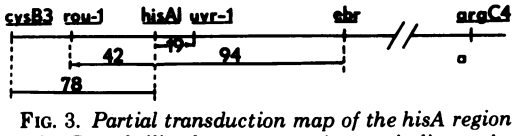

FIG. 3. *Partial transduction map of the hisA region on the B. subtilis chromosome. Arrows indicate the direction of selection of the crosses, with direct selection being made for the marker at the tail of the arrow. No arrow represents the average recombination distance from crosses in both directions. a, Recombination distance "78" was taken from Dubnau et al. (5).*

DISCUSSION

Our results demonstrate that expression of resistance in an EB-resistant mutant of *B. subtilis* (EB8) is conditional. Resistance to EB is expressed during vegetative growth in CA-supplemented MG or complex media and at incubation temperatures greater than 30 C, suggesting that a cell component is possibly altered in configuration or is not synthesized, or that a new component is synthesized under these conditions. Outgrowing spores become at least partially resistant to EB at about 45 min after the start of germination, indicating that at this

TABLE 3. *Analysis of three-factor transduction crosses*[a]

Recipient and selected phenotype	*hisA1*	*rou-1*	*ebr*	*uvr-1*	No. of recombinants
BR19, His$^+$[b]	1[c]	1	1		2
	1	1	0		329
	1	0	1		57
	1	0	0		184
GSY1057, His$^+$[d]	1		1	1	35
	1		0	1	372
	1		1	0	1
	1		0	0	93
GSY1057, EB^r[e]	1		1	1	29
	0		1	1	39
	1		1	0	0
	0		1	0	545

[a] Donor: PBS1 lysate prepared on strain EB8.
[b] Probable order: *rou-1, hisA1, ebr.*
[c] Donor and recipient phenotypes are denoted by 1 and 0, respectively.
[d] Probable order: *hisA1, uvr-1, ebr.*
[e] Probable order: *ebr, uvr-1, hisA1.*

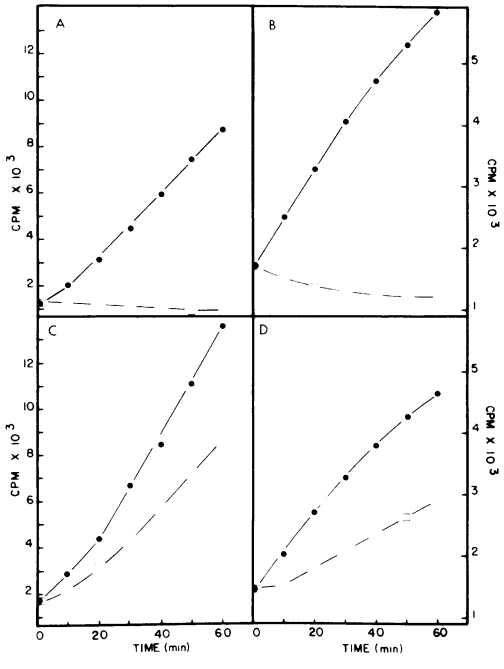

FIG. 4. *Effect of EB on RNA synthesis, as measured by [5-^{3}H] uridine uptake into acid-insoluble material, in whole cells and protoplasts of Marburg and EB8. A, Marburg whole cells. B, Marburg protoplasts. C, EB8 whole cells. D, EB8 protoplasts. Symbols: ● and □ represent 0 and 10 μg of EB per ml, respectively.*

point some cell component is synthesized or can accumulate in sufficient quantities such that during outgrowth it can counteract the complete inhibitory effect of EB. Since sporulation is blocked by EB, the cell component is either not present, is altered, or fails to protect cells at sometime during late-log or early-stationary growth phase. However, at least one cellular function that occurs during late-log phase was not blocked, namely cell motility.

The location of the EB resistance marker in the *hisA* region of the *B. subtilis* chromosome is close to genes known to regulate cell wall synthesis (3, 23) and cell division (21). However, RNA synthesis by protoplasts is resistant to EB, implying that a modified cell wall is probably not the major cause of EB resistance in strain EB8.

Fluorescence measurements of protoplasts of the Marburg and EB8 strains, suspended in high-sucrose media containing EB, failed to reveal any difference in the enhancement of EB fluorescence by the two strains and indicates that the binding of EB to protoplasts of the two strains is probably qualitatively and quantitatively very similar. This result does not rule out an alteration in the cell membrane. Disruption of the protoplasts by dilution with distilled water enhanced the fluorescence of EB to about the same extent in both strains, which would decrease the probability that the nucleic acids of strain EB8 have been altered in their ability to bind EB. Binding differences were shown by Saffhill et al. (18) when they compared the enhancement of fluorescence of EB with RNA from an EB-resistant variant of QB bacteriophage with that of a sensitive variant.

Three possible explanations for EB resistance are: (i) mutational alteration of EB-stimulated deoxyribonuclease activity, an enzyme activity reported by Paoletti et al. (15) in yeast mitochondria; (ii) alteration of deoxyribonucleic acid or RNA polymerase sensitivites, or both, as suggested by Bouanchaud et al. (2); and (iii) modification of the cell membrane as has been shown by Nakamura and Suganuma (14) in the case of an acriflavine-sensitive mutant of *Escherichia coli* K-12. The phenotype of strain EB8 appears to be most consistent with the latter explanation.

At the present time, the mode of EB resistance in strain EB8 is not known and is under investigation.

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