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The Biological Effect of Evernimicin B on Bacillus Subtilis W2 3 and Three Antibiotic-Resistant Strains of Staphylococcus Aureus

Steven Bogach
Western Kentucky University

Bogach,

Steven Joel

1981

THE BIOLOGICAL EFFECT OF EVERNINOMICIN B ON *BACILLUS SUBTILIS* W23 AND THREE ANTIBIOTIC-RESISTANT STRAINS OF *STAPHYLOCOCCUS AUREUS*

A Thesis

Presented to

the Faculty of the Department of Biology

Western Kentucky University

Bowling Green, Kentucky

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

Steven Joel Bogach

June, 1981

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Date July 7, 1989

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THE BIOLOGICAL EFFECT OF EVERNINOMICIN B ON BACILLUS SUBTILIS W23 AND THREE ANTIBIOTIC-RESISTANT STRAINS OF STAPHYLOCOCCUS AUREUS

Recommended July 7, 1981
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Approved July 14, 1981
(Date)

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Dean of the Graduate College

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This thesis is dedicated to my wife, Margaret Jane Bogach, whose love and unerring faith in my abilities made this accomplishment possible. We did it, Sweetheart.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

THE BIOLOGICAL EFFECT OF EVERNINOMICIN B ON *BACILLUS SUBTILIS* W23 AND THREE ANTIBIOTIC-RESISTANT STRAINS OF *STAPHYLOCOCCUS AUREUS*

Steven J. Bogach June, 1981 38 pages

Directed by: Rebecca A. Brown, M. R. Houston, E. J. Hoffman, and J. R. McCurry

Department of Biology Western Kentucky University

One strain of *Bacillus subtilis* and three antibiotic-resistant strains of *Staphylococcus aureus* were employed to determine the biological effects of everninomicin B (EvB), a naturally occurring antibiotic produced by *Micromonospora carbonacea* (NRRL 2972) and *M. carbonacea* var. *aurantiaca*. Minimum inhibitory concentration (MIC) of EvB for *B. subtilis* was 1.2×10^{-3} µmole/ml of EvB in glucose minimal broth and 2.6×10^{-3} µmole/ml of EvB in nutrient broth. MIC values of EvB for *S. aureus* were 3.25×10^{-4} µmole/ml of EvB for strains resistant to penicillin or tetracycline and 6.5×10^{-4} µmole/ml of EvB for the aminoglycoside-resistant strain. The inhibitory effect of EvB was found to be reversible for all concentrations of EvB and all bacterial strains tested. The inhibitory effect of EvB for *B. subtilis* was not dependent upon the initial concentration of cells nor the stage in the growth cycle at the time the compound was introduced. The inhibitory effect was dependent upon the initial concentration of cells for *S. aureus*. Electron microscopy studies showed distinct morphological changes in treated cells of *S. aureus*. Cellular lysis in these cells was also detected.

INTRODUCTION

In 1904 Ehrlich first described the feasibility of using detoxified poisons (such as arsenic) to treat infections. Since then researchers have been isolating and chemically synthesizing many types of antimicrobial agents. In 1929 Fleming noticed that certain fungal metabolites would inhibit bacterial growth. These fungal metabolites were termed antibiotics (Rogers, 1969).

There are at least five modes of action by which antibiotics inhibit bacterial growth: (1) interruption of necessary biochemical pathways, (2) inhibition of cell wall synthesis, (3) inhibition of DNA or RNA synthesis, (4) inhibition of protein synthesis, and (5) physical damage to the cell membrane (Richmond, 1966).

The first and most important antibiotic to be used was penicillin. Penicillin is a naturally occurring antibiotic produced by the fungus Penicillium notatum and is one of many antibiotics which inhibit peptidoglycan (cell wall) synthesis (Park and Strominger, 1957). When the cell wall cannot be synthesized the osmotically fragile protoplast will lyse (Hancock and Fitz-James, 1964).

When antibiotics became commonly used, antibiotic-resistant bacteria were selected for and became a major medical problem. Antibiotic-resistant bacteria are those bacteria with the genetic ability to resist the

effects of an antibiotic (Kazuo et al., 1966). As the types and strains of antibiotic-resistant bacteria increased, the need for new and effective antibiotics became evident.

The genus Micromonospora produces five families of clinically beneficial antibiotics (Table 1) and several antibiotics which have been isolated but not fully described (Wagman and Weinstein, 1980). The aminoglycosides are the most prominent of the antibiotics produced by Micromonospora. The most clinically utilized aminoglycoside is gentamicin which is a water-soluble antibiotic synthesized by M. purpurea that has an unusually broad spectrum and can inhibit the growth of antibiotic-resistant bacteria such as Proteus and Pseudomonas (Wagman and Weinstein, 1980; Rosselet et al., 1964; Oden et al., 1964). Its broad spectrum and low toxicity for the host organism makes it an excellent antibiotic of choice against gram-negative bacteria (Wagman and Weinstein, 1980). Gentamicin is known to bind irreversibly to the 30S ribosomal subunit which prevents translation during protein synthesis (Petska, 1971).

The macrolides produced by Micromonspora can inhibit bacterial growth only at an alkalinr pH and have mainly a gram-positive spectrum (Wagman and Weinstein, 1980). The macrolides inhibit bacterial growth by binding to the 50S ribosomal subunit which prevents protein synthesis (Petska, 1971.

The ansamysins have been isolated from many species of Micromonspora. Included in this family are the halomycins and rifamicins (Weinstein et al., 1968). The ansamycins prevent bacterial growth by inhibiting DNA-dependent Rna polymerase thus preventing RNA synthesis. Mammalian cells have been found to contain a RNA polymerase insensitive to rifamicins , and the cells are not known to be affected (Wehrli and Staehelin, 1971).

Actinomysins are peptide containing antibiotics with a broad spectrum and a high degree of host toxicity (Reich, 1964). These antibiotics are the most potent and specific inhibitiors of DNA-dependent RNA synthesis (Gellert et al., 1965). The actinomycins have been used to study viral replication and RNA function in prolaryotic and eukaryotic systems (Reich et al., 1962). Kirk (1962) showed that in mammalian systems, the actinomycins form reversible complexes with DNA. The modes of action of the actinomycins are: (1) prevention of DNA polymerization, (2) condensation of chromatin, and (3) the loss of nuclear functions which require the physical involvement of DNA (Reich et al., 1962).

The everninomicins are a family of five related antibiotics designated A, B, C, D, and E. The are naturally synthesized by M. carbonacea (NRRL 2972) and M. carbonacea var. aurantiaca (Wagman et al., 1965). The everninomicins are composed of a dichloroseverinic acid complexed with a polysacharide moeity (Herzog et al., 1965). Pharmacological

Table 1.

Antibiotics Produced by Micromonospora

Family	Generic Name of Antibiotic
AMINOGLYCOSIDES	Gentamicins
	Sisomycin
	Verdamicin
	Antibiotic G-52
	Fortimicins
	Neomycins
	Antibiotic 460
	Sagamicin
MACROLIDES	Megalomicins
	Rosaramicin
	Juvenimicins
	M-4365 Complex
	Erythromycin B
ANSAMYSINS	Halomycins
	Rifamicins
EVERNINOMICINS	Everninomicin A
	Everninomicin B
	Everninomicin C
	Everninomicin D
	Everninomicin E
ACTINOMYCINS	Actinomycin C
	Actinomycin D
MISCELLANEOUS ANTIBIOTICS*	

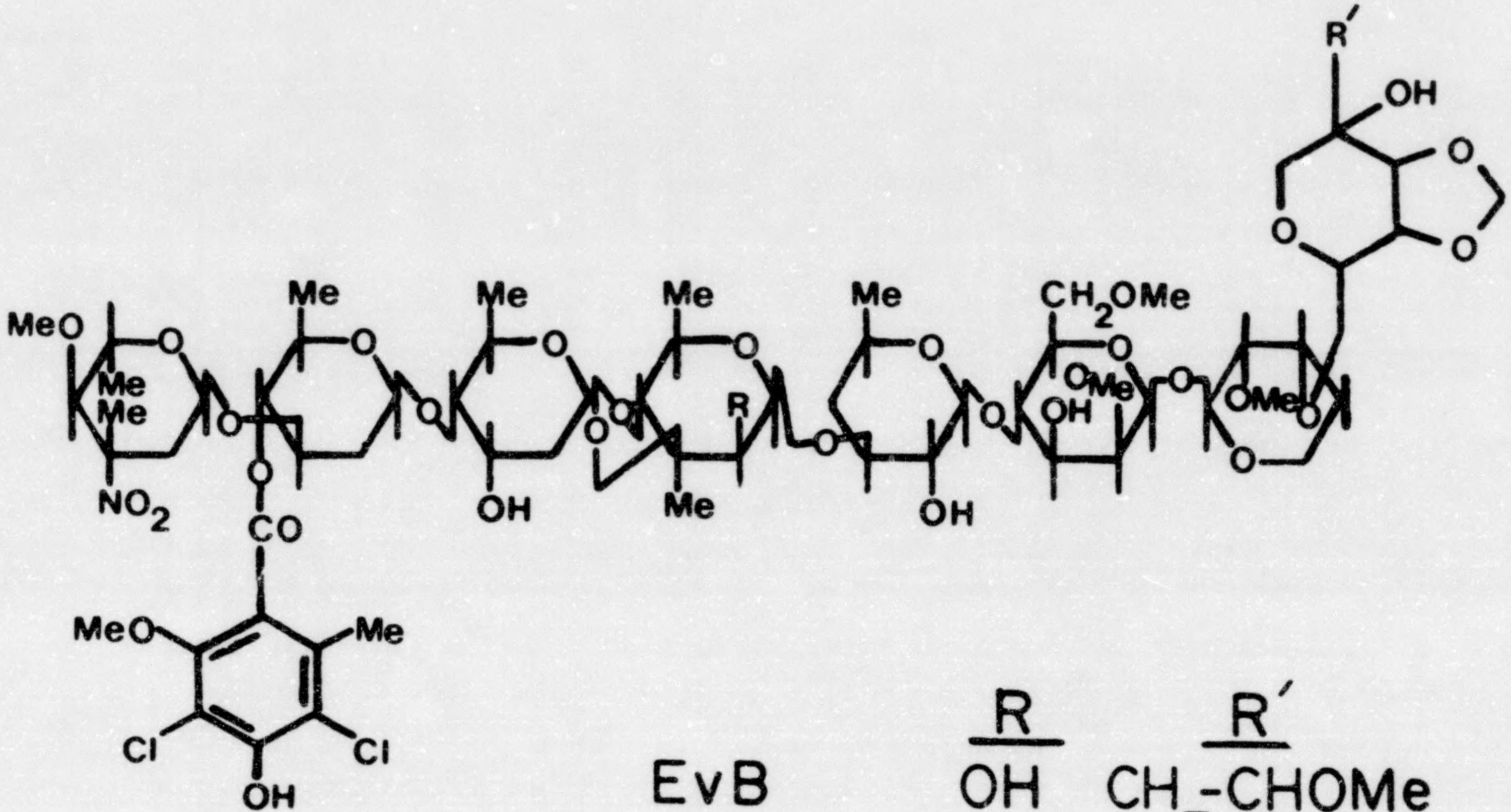
*Isolated but not yet fully described.

research with everninomicin D (EvD) showed that EvD had extremely low host toxicity. In mice, EvD was shown to have a LD_{50} of 3700 µg/kg when injected subcutaneously and 125 µg when injected intravenously (Weinstein, et al., 1965). Their study showed that when EvD was administered subcutaneously it would provide in vivo protection for mice against lethal strains of Staphylococcus aureus, Steptococcus pyogenes, and Diplococcus pneumoniae. The amount needed for complete protection against these bacterial infections was comparable to penicillin G, methicillin, or oxacillin (Weinstein, et al., 1965).

Black, et al., (1965) also researched the pharmacological effect of EvD. In their study they utilized mice, dogs (beagle hounds), and human beings. Their results showed that EvD readily binds to human serum (94%). The rapidity of serum binding by the antibiotic greatly diminished the antimicrobial effect. In addition, subcutaneous administration resulted in poor adsorption in muscle and the compound was excreted via urinary and biliary routes. Other problems included finding a suitable vehicle for injection, eliminating soreness at the site of injection, and overcoming a distinct organic solvent taste when administered orally.

Sanders and Sanders (1978) showed that EvB (which is structurally similar to EvD) (Figure 1) had primarily a gram-positive spectrum. Sensitive gram-negative bacteria are in the genera Bacterioides, Hemophilus, and Neisseria. They also

Figure 1. Structure of Everninomicin B and Everninomicin D.



	R	R′
EvB	OH	CH_3-CHOMe
EvD	H	CH_3-CHOMe

showed that 73 strains of antibiotic-resistant gram-positive bacteria were sensitive to EvB. Their results indicated that there was no de novo resistance in any of the bacteria tested. Because antibiotic-resistant bacteria are sensitive to EvB and no resistance to the compound has been demonstrated, it was suggested that EvB may have a novel mode of action not previously described (Sanders and Sanders, 1978).

The objectives of this project were to (1) find the minimum inhibitory concentration of EvB needed to inhibit the growth of Bacillus subtilis W23 and three antibiotic-resistant strains of Staphylococcus aureus, (2) determine the reversibility of EvB-induced inhibition for Bacillus subtilis W23 and three antibiotic-resistant strains of Staphylococcus aureus, (3) determine if the effect of EvB is dependent upon the initial concentration of cells of actively growing cultures of Bacillus subtilis W23 and penicillin-resistant Staphylococcus aureus, (4) determine the effect of EvB on the viability of Bacillus subtilis W23 and penicillin-resistant Staphylococcus aureus, and (5) visualize any physical effects that EvB might have upon the penicillin-resistant Staphylococcus aureus using electron microscopy.

MATERIALS AND METHODS

Preparation of Everninomicin B for Bacterial Testing

The compound used in this study was everninomicin B (EvB) originally obtained from the Schering Corporation, Bloomfield, New Jersey. EvB, a white amorphous powder, was kept in a screw-top vial and stored at 5°C. A stock solution was prepared by placing 0.005 g of EvB and 0.5 ml of dimethylsulfoxide (DMSO) into a sterile cap-all test tube. Sterile distilled water (4.5 ml) was then added bringing the final concentration of the solution to 0.63 µmole of EvB/ml. Stock solutions of EvB were stored in cap-all test tubes at 5°C. For experimentation appropriate dilutions were made of the stock solutions.

Microorganisms

Bacillus subtilis W23 was obtained from the collection of Dr. R. A. Brown, Western Kentucky University, Bowling Green, Kentucky. Stock cultures of *B. subtilis* W23 were maintained on glucose minimal agar slants at 5°C.

Three strains of antibiotic-resistant *Staphylococcus aureus* (wild strains) were clinically isolated at Greenview Hospital, Bowling Green, Kentucky. The three antibiotic-resistant strains employed were resistant to either penicillin, tetracycline, or aminoglycosides. All three strains were found to be gram-positive cocci in clusters, catalase positive,

coagulase positive, and positive for mannitol fermentation. The S. aureus strains were maintained on slants of brain heart infusion agar at 5°C.

Bacterial Culture Media

Glucose minimal medium used in this study was composed of 0.2% (w/v) NaCl, 0.2% (w/v) $(NH_4)_2SO_4$, 0.32% (w/v) KH_2PO_4, and 5% (w/v) glucose. The basal medium was prepared by dissolving the NaCl, $(NH_4)_2SO_4$, and KH_2PO_4 in deionized distilled water and adjusting the pH to 7 with 1M KOH. The basal medium was autoclaved at 121°C for 15 minutes. Prior to use the basal medium was cooled to 25°C. A stock solution of 10% (w/v) glucose and a mineral salts solution were prepared and autoclaved separately. The mineral salts solution was composed of 5% (w/v) $MgSO_4 \cdot 7H_2O$, 1% (w/v) $MnSO_4$, and 0.5% (w/v) $CaCl_3$ in deionized distilled water. The basal medium was supplemented with 0.1 ml mineral salts solution for each 100 ml basal medium, and glucose added to a final concentration of 5% (v/v). Agar (DIFCO) was added to a final concentration of 1.5% (w/v) for solid medium.

Brain heart infusion broth and brain heart infusion agar (DIFCO) were used in the cultivation of S. aureus. These media were made and sterilized in accordance with label instructions.

Tube Dilution

The tube-dilution protocol was used to determine the minimum inhibitory concentration (MIC) of EvB for the bacteria tested. Twelve test tubes were used for each

bacterium tested. One ml of the appropriate broth was dispensed into each test tube except for the first test tube. Two ml of appropriate broth containing 2.1×10^{-2} µmole/ml of EvB were added to the first test tube. Serial two-fold dilutions were made starting from 2.1×10^{-2} µmole of EvB/ml and ending with 1.05×10^{-5} µmole of EvB/ml.

Bacteria from the stock cultures were inoculated into 9 ml of sterile broth and incubated for 18 hours at 36°C on a rotary shaker. The broth culture was diluted 1:100 and one ml was dispensed into each of the 12 test tubes. The final concentrations of EvB after the addition of inoculum were 1.05×10^{-2}, 5.2×10^{-3}, 2.6×10^{-3}, 1.3×10^{-3}, 6.5×10^{-4}, 3.25×10^{-4}, 1.63×10^{-4}, 8.13×10^{-5}, 4.10×10^{-5}, 2.03×10^{-5}, 1.02×10^{-5}, and 5.08×10^{-6} µmole/ml, respectively. The twelve test tubes were incubated for 18 hours at 36°C on a rotary shaker and checked for visible growth. The highest dilution without visible growth was the MIC.

Reversibility was determined by removing 0.1-ml aliquots from all cultures showing no growth in each series and inoculating them into 5 ml of sterile broth. These subcultures were then incubated for 18 hours at 36°C on a rotary shaker. After incubation the culture test tubes were checked for visible growth. The highest dilution without visible growth was considered to be the minimum bacteriocidal concentration (MBC). If all the tubes showed visible growth, the effect of the compound was considered to be reversible.

Growth Experiments

Stock culture from an agar slant was loop inoculated into 9 ml of sterile broth and then incubated for 18 hours at 36°C on a rotary shaker. These 18-hour cultures were then used as inocula for further experimentation. For growth studies, cells were inoculated into sterile test tubes (150 x 15 mm) containing 5.0 ml sterile broth. Two growth controls were used in all growth studies. Control A was made by adding only sterile distilled water and control B by adding only DMSO stock solution to the test tube. Added to the experimental test tubes were appropriate concentrations of EvB. All test tubes were inoculated to the desired optical density (between 0.06 and 0.08 units). Cell growth was monitored spectrophotometrically at 540 nm using a Bausch and Lomb Spectronic 20 with an 18-mm light path. Growth was monitored at hourly intervals for 8 hours with a final reading at 24 hours.

Viability Determinations

Two side-arm flasks containing 25 ml of culture broth were inoculated to an optical density of 0.10 units. To the control flask, only sterile distilled water was added. EvB was placed into the second side-arm flask at a concentration of 1.3×10^{-2} µmole/ml. Both flasks were incubated at 36°C until the optical density in the control flask reached 0.13 units. At designated times, one-ml aliquots from each side-arm flask were placed into separate 99-ml dilution blanks containing potassium phosphate buffer (pH 7.2). These dilution blanks were then shaken (25 one-foot strokes in 7 seconds) and

serial ten-fold dilutions were made starting from 10^{-2} and ending with 10^{-9}. Each dilution was plated in duplicate using the appropriate agar and incubated for 24 hours at 36°C. Those plates showing between 30 and 300 colonies were counted using a Quebec Colony Counter.

Electron Microscopy

A 0.5% (w/v) solution of formvar in chloroform was prepared and allowed to stand at 25°C for 24 hours. A microscope slide was immersed in the formvar solution, and the chloroform was allowed to evaporate. Using a new razor blade, a series of slices were made on the periphery of the slide to initially release the formvar which was then loosened by gently blowing on the slide. The loosened formvar was then floated onto the surface of clean distilled water in a 500-ml culture dish. Copper grids were placed onto the floating formvar sheet, and a pre-cleaned glass slide was placed over the formvar sheet and grids. This slide was removed, inverted and allowed to dry overnight at 25°C in a dust-free environment.

Cells of S. aureus were treated with 1.3×10^{-3} µmole of EvB per ml and allowed to incubate for 18 hours at 36°C on a rotary shaker. Controls were grown in brain heart infusion broth in which sterile distilled water was substituted for EvB and incubated for 18 hours at 36°C on a rotary shaker. All cells were centrifuged at 10,000 x g for 15 minutes in a Sorval 55-1 centrifuge. The supernatant was discarded and the cells washed three times in potassium phosphate buffer

(pH 7.2). After washing the cells were dropped onto formvar grids with a pasteur pipet. A drop of 7% uranyl acetate was placed onto the grids with the cells for negative staining. The formvar grids were then blotted to dryness on cellulose filter-paper disks, and the grids were observed with a Zeiss EM 9S-2 electron microscope.

RESULTS

Minimum Inhibitory Concentrations

The MIC of EvB when using Bacillus subtilis W23 grown in glucose minimal broth was 1.3×10^{-3} µmole of EvB per ml. The MIC of EvB when B. subtilis W23 was grown in nutrient broth was 2.6×10^{-3} µmole of EvB per ml. This represented a two-fold increase in MIC (Table 2) when the media were changed.

The MIC of EvB for the three antibiotic-resistant strains of S. aureus was 3.25×10^{-4} µmole of EvB per ml for the penicillin-resistant strain and the tetracycline-resistant strain and 6.5×10^{-4} µmole of EvB per ml for the strain of S. aureus resistant to aminoglycosides (Table 2).

Reversibilities

The reversibility of EvB-induced inhibition was determined for all bacterial strains used in this study. B. subtilis W23 was subcultured into fresh medium from all negative tubes. For cells grown in glucose minimal broth this represented concentrations of EvB starting from 1.3×10^{-3} to 1.05×10^{-2} µmole/ml. When grown in nutrient broth, the concentrations of EvB from the negative growth cultures ranged from 2.6×10^{-3} to 1.05×10^{-2} µmole/ml. The results indicated that the effect of the compound was reversible for B. subtilis W23 for all concentrations of EvB tested (Table 3).

TABLE 2

MINIMUM INHIBITORY CONCENTRATION DETERMINATIONS FOR *BACILLUS SUBTILIS* W23 AND THREE ANTIBIOTIC-RESISTANT STRAINS OF *STAPHYLOCOCCUS AUREUS*

µmole of EvB/ml	*S. aureus*[a]			*B. subtilis*[b]	
	P-R	T-R	A-R	NB	GMMB
1.05×10^{-2}	-[c]	-	-	-	-
5.20×10^{-3}	-	-	-	-	-
2.60×10^{-3}	-	-	-	-	-
1.30×10^{-3}	-	-	-	+[d]	-
6.50×10^{-4}	-	-	-	+	+
3.25×10^{-4}	-	-	+	+	+
1.63×10^{-4}	+	+	+	+	+
8.13×10^{-5}	+	+	+	+	+
4.10×10^{-5}	+	+	+	+	+
2.03×10^{-5}	+	+	+	+	+
1.02×10^{-5}	+	+	+	+	+
5.08×10^{-6}	+	+	+	+	+

[a] P-R: penicillin-resistant strain
T-R: tetracycline-resistant strain
A-R: aminoglycoside-resistant strain

[b] NB: *B. subtilis* grown in nutrient broth
GMMB: *B. subtilis* grown in glucose minimal broth

[c] negative growth

[d] positive growth

TABLE 3

REVERSIBILITY OF EVERNINOMICIN-B-INDUCED INHIBITION FOR *BACILLUS SUBTILIS* W23 AND THREE ANTIBIOTIC-RESISTANT STRAINS OF *STAPHYLOCOCCUS AUREUS*

µmole of EvB/ml	*S. aureus*[a]			*B. subtilis*[b]	
	P-R	T-R	A-R	NB	GMMB
1.05×10^{-2}	+[c]	+	+	+	+
5.20×10^{-3}	+	+	+	+	+
2.60×10^{-3}	+	+	+	+	+
1.30×10^{-3}	+	+	+	+	+
6.50×10^{-3}	+	+	+	+	+

[a] P-R: penicillin-resistant strain
T-R: tetracycline-resistant strain
A-R: aminoglycoside-resistant strain

[b] NB: *B. subtilis* grown in nutrient broth
GMMB: *B. subtilis* grown in glucose minimal broth

[c] positive growth

All negative cultures from antibiotic-resistant S. aureus were subcultured into fresh brain heart infusion broth. The concentrations of EvB of the negative growth cultures ranged from 3.25×10^{-4} to 1.05×10^{-2} μmole/ml for the penicillin- and tetracycline-resistant strains and 6.5×10^{-4} to 1.05×10^{-2} μmole/ml for the strain resistant to aminoglycosides. The results indicate that the effect of the compound was reversible for all concentrations of EvB tested for the three antibiotic-resistant strains (Table 3).

Growth Studies

B. subtilis W23 was inoculated into glucose minimal broth with EvB at a concentration of 6.5×10^{-3} μmole/ml which represented a five-fold increase of the MIC value. There was 100% growth inhibition after 24 hours (Figure 2). Penicillin-resistant S. aureus was grown in brain heart infusion broth with EvB added at concentrations of 1.3×10^{-3} and 6.5×10^{-3} μmole/ml. Both concentrations of EvB had the same effect, 66% growth inhibition when compared to the controls (Figure 3).

After the initial growth studies were performed, the stability of compound was investigated to determine if the effects of incubation (i.e. shaking, temperature, degradation of compound due to media, or incubation time) could result in the loss of biological activity of EvB. Three series of test tubes containing glucose minimal broth plus 1.3×10^{-2}

Figure 2. Effect of Everninomicin B on growth of Bacillus subtilis W23 (●), glucose minimal broth controls (■), 6.5×10^{-3} µmole/ml of EvB in glucose minimal broth.

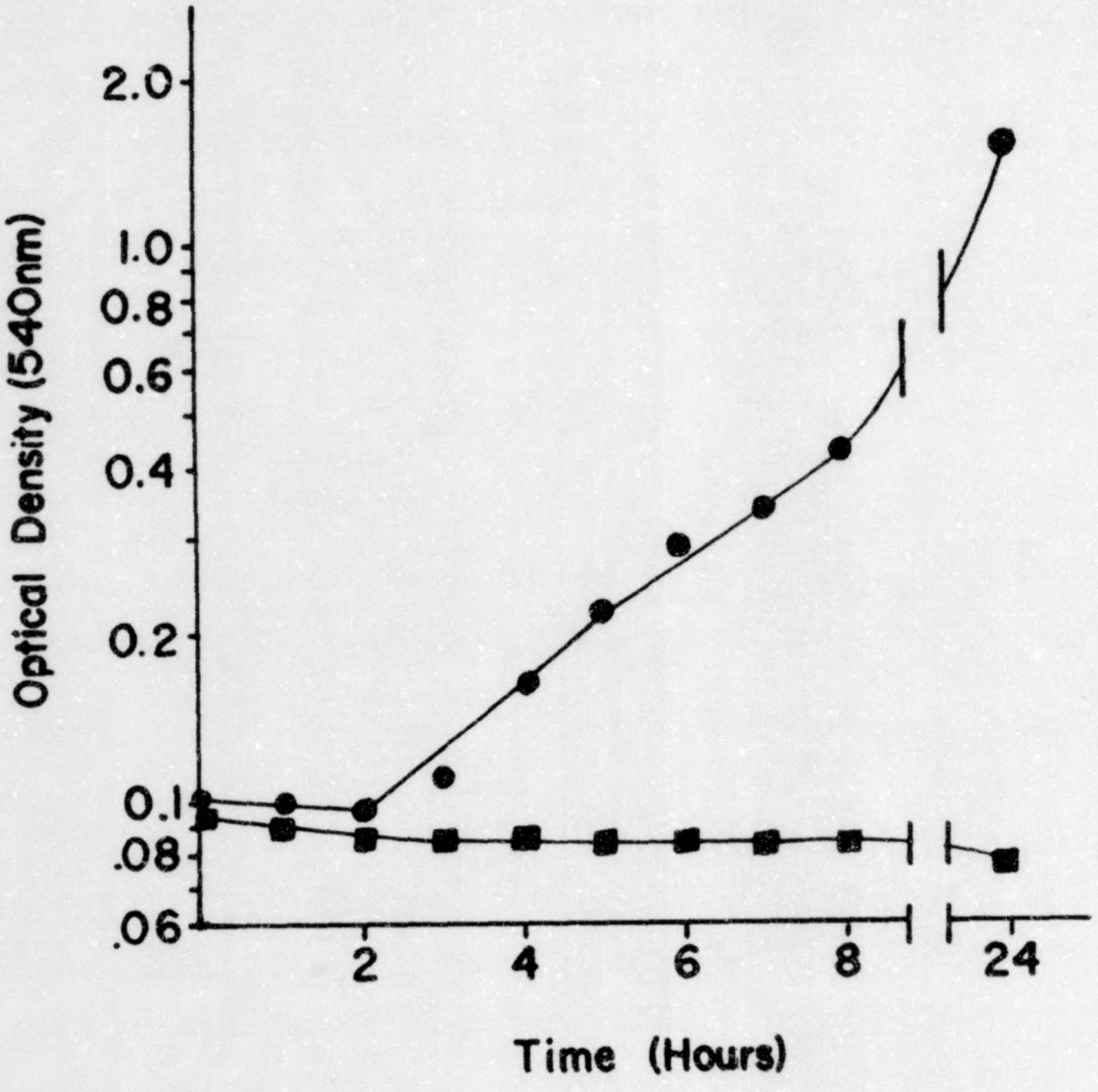
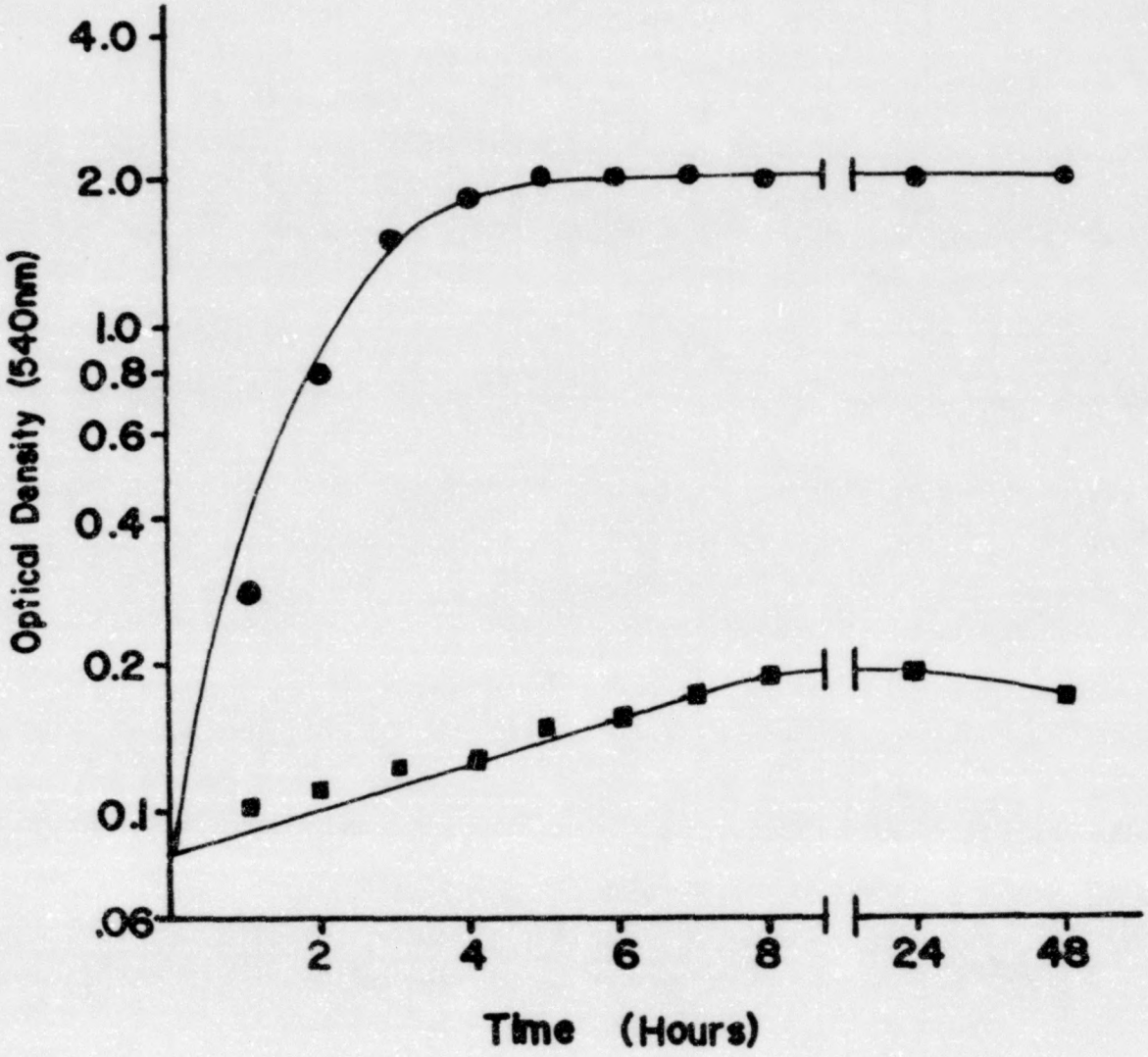

Optical Density (540nm)
2.0
1.0
0.8
0.6
0.4
0.2
0.1
.08
.06
2
4
6
8
24
Time (Hours)

Figure 3. Effect of Everninomicin B on the growth of penicillin-resistant Staphylococcus aureus (●), brain heart infusion controls (■), 6.5×10^{-3} and 1.3×10^{-2} μmole/ml of EvB in brain heart infusion broth.

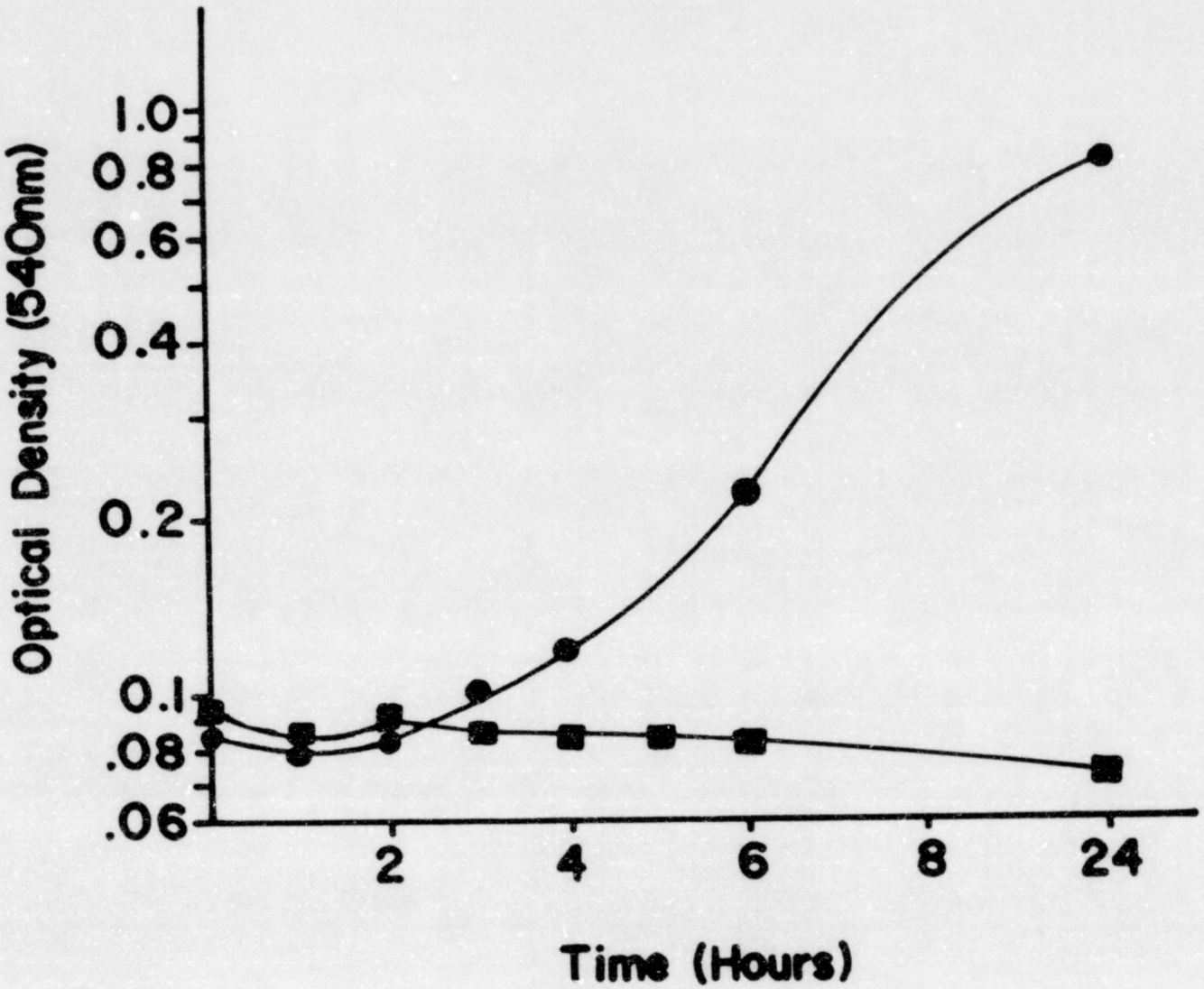

4.0
2.0
1.0
0.8
0.6
0.4
0.2
0.1
.06
Optical Density (540nm)
2
4
6
8
24
48
Time (Hours)

μmole/ml of EvB and three control series were incubated cell-free on a rotary shaker at 36°C for 24, 48, and 72 hours. After each 24-hour cell-free incubation period, B. subtilis W23 was inoculated into the EvB and control series which were then incubated for an additional 24 hours at 36°C on a rotary shaker. The results showed that after 72 hours of standard cell-free incubation, EvB did not lose its ability to inhibit the growth of B. subtilis W23 (Figure 4).

Studies of the effect of initial inoculum size were used to determine if the inhibitory effect of EvB was dependent upon the initial concentration of cells in the culture medium. B. subtilis W23 was inoculated into four series of test tubes containing glucose minimal broth and EvB at a concentration of 6.5×10^{-3} μmole/ml. The initial optical densities in the four series were 0.2, 0.2, 0.3, and 0.4 units. Control test tubes were run for each series and adjusted to comparable optical densities. B. subtilis W23 showed 100% inhibition after 24 hours at all optical densities employed (Figure 5).

The effect of initial inocula size was determined using penicillin-resistant S. aureus. All growth conditions were kept similar to the previous experiment except that brain heart infusion medium was used. At an initial optical density of 0.1 units S. aureus treated with EvB showed 50% growth inhibition after 24 hours. When S. aureus was inoculated to an initial optical density of 0.2 units the treated cells

Figure 4. Effect of growth conditions on the stability of Everninomicin B after 72 hours cell-free incubation (●), glucose minimal broth controls; (■), 1.3 x 10^{-2} µmole/ml of EvB in glucose minimal broth.


Optical Density (540nm)
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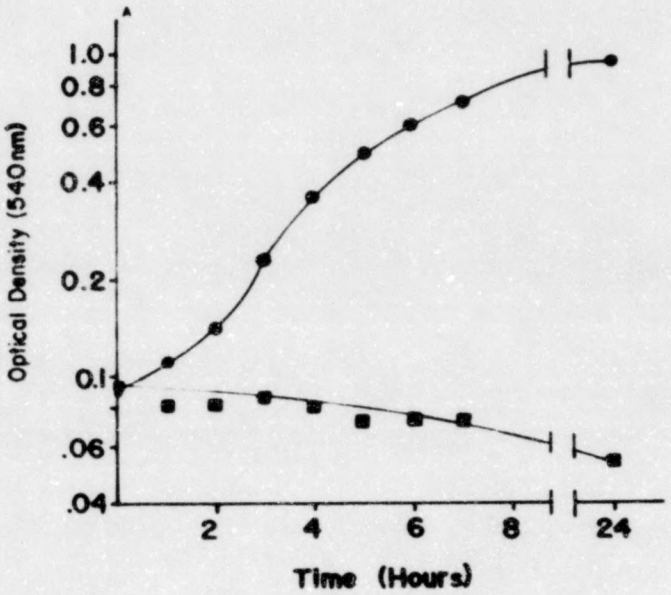
0.8
0.6
0.4
0.2
0.1
.08
.06
2
4
6
8
24
Time (Hours)

Figure 5. Effect of initial inoculum size on Everninomicin B induced growth inhibition of Bacillus subtilis W23. A: initial optical density 0.1 OD units, B: initial optical density 0.2 OD units, C: initial optical density 0.3 units, D: initial optical density 0.4 OD units. (●), glucose minimal broth controls; (■), 6.5×10^{-3} µmole/ml of EvB in glucose minimal broth.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Optical Density (540nm)
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0.2
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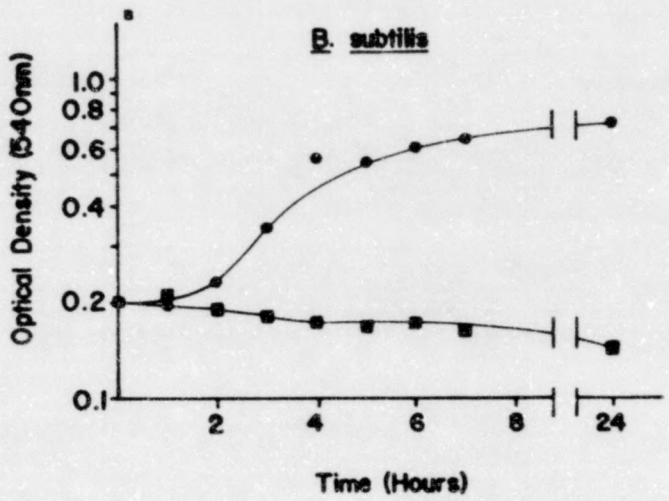
2
4
6
8
24
Time (Hours)


B
B. subtilis
Optical Density (540nm)
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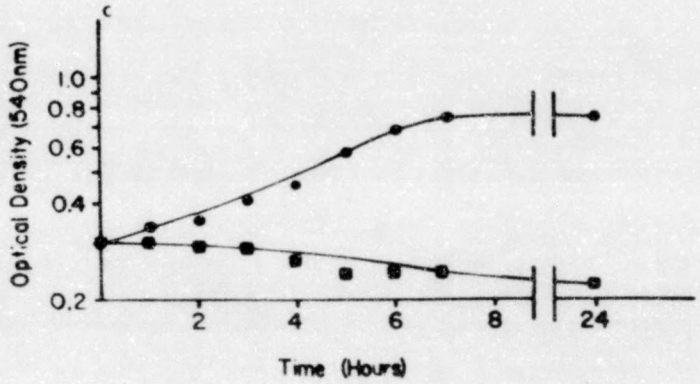
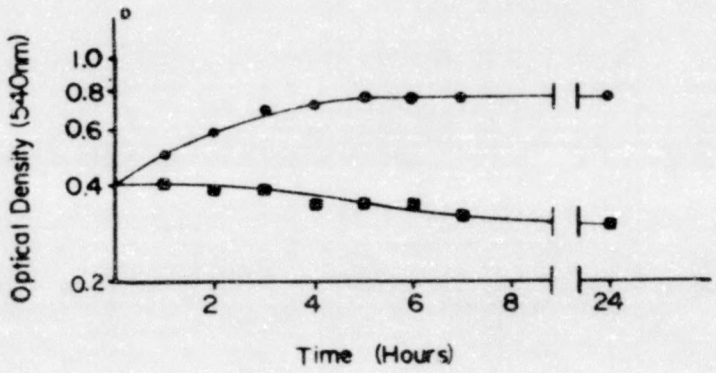
0.8
0.6
0.4
0.2
0.1
2
4
6
8
24
Time (Hours)


C
Optical Density (540nm)
1.0
0.8
0.6
0.4
0.2
2
4
6
8
24
Time (Hours)

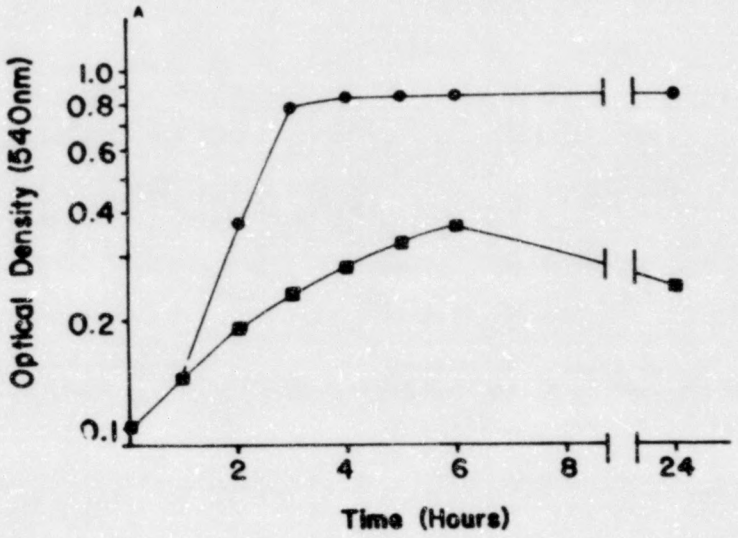

D
Optical Density (540nm)
1.0
0.8
0.6
0.4
0.2
2
4
6
8
24
Time (Hours)

showed approximately 15% growth inhibition after 24 hours. At initial optical densities of 0.3 and 0.4 units there was no EvB-induced inhibition after 24 hours for treated cells of S. aureus. These results indicated that the effect of EvB was dependent upon initial concentration of cells for S. aureus (Figure 6).

Time-of-addition studies were performed to determine if EvB had more or less of an inhibitory effect when added at different times during the incubation period. B. subtilis W23 was inoculated into glucose minimal broth at an optical density of 0.08 units and incubated on a rotary shaker at 36°C until a change in optical density was detected. EvB was added at a concentration of 6.5×10^{-3} µmole/ml to the actively growing cultures at 2-hour intervals. As the compound was added, sterile distilled water and DMSO were added to the controls. The results showed that when EvB was added at any time during the incubation period there was 100% inhibition of growth (Figure 7).

Time-of-addition studies were performed using penicillin-resistant S. aureus. The growth conditions were similar to the previous experiment except that shorter addition times were used, and brain heart infusion broth was the medium of choice. These studies showed that when EvB was added initially there was 50% growth inhibition after 24 hours and no inhibition after 48 hours. When the compound was added after one hour to actively growing cultures, there was also 50% growth

Figure 6. Effect of initial inoculum size on Everninomicin B induced growth inhibition of penicillin-resistant Staphylococcus aureus. A: initial optical density 0.1 OD units, B: initial optical density 0.2 OD units, C: initial optical density 0.3 OD units, D: initial optical density 0.4 OD units. 10^{-3} (●), glucose minimal broth controls; 6.5 x 10^{-3} µmole/ml of EvB in brain heart infusion broth■.


A
Optical Density (540nm)
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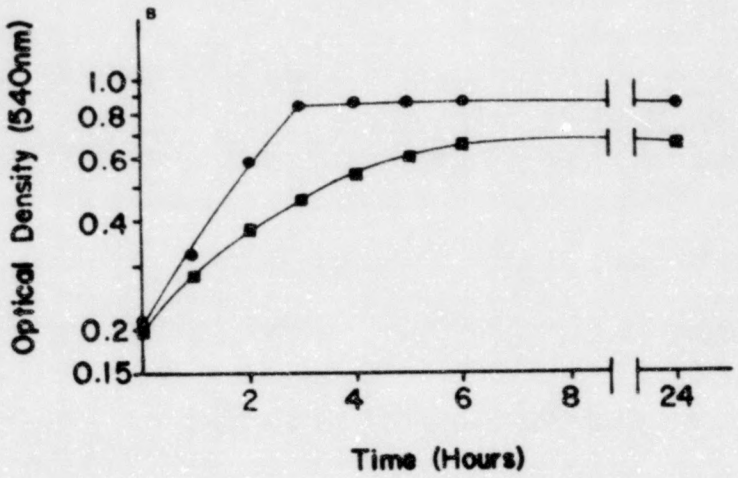
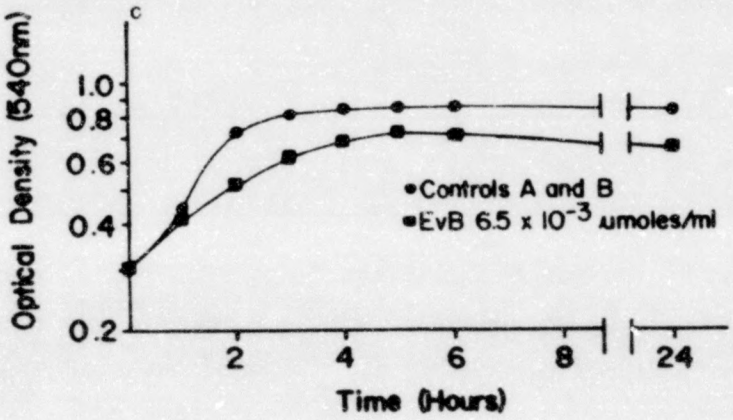
0.8
0.6
0.4
0.2
0.1
2
4
6
8
24
Time (Hours)


B
Optical Density (540nm)
1.0
0.8
0.6
0.4
0.2
0.15
2
4
6
8
24
Time (Hours)


C
Optical Density (540nm)
1.0
0.8
0.6
0.4
0.2
•Controls A and B
•EvB 6.5 x 10⁻³ µmoles/ml
2
4
6
8
24
Time (Hours)

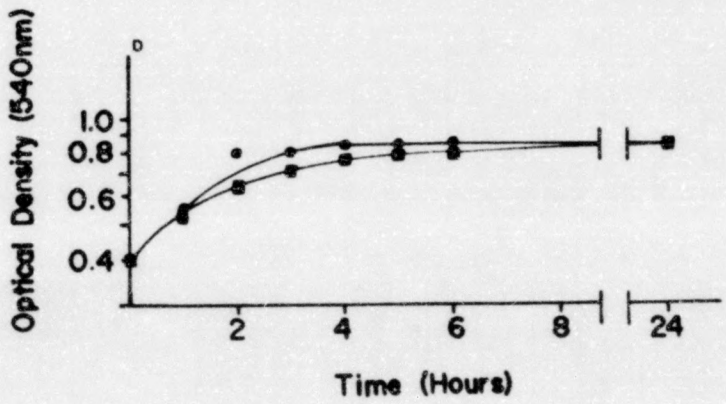
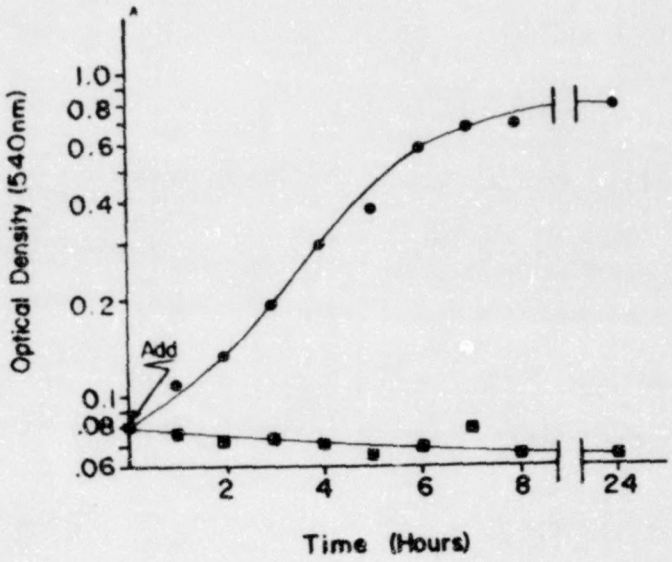
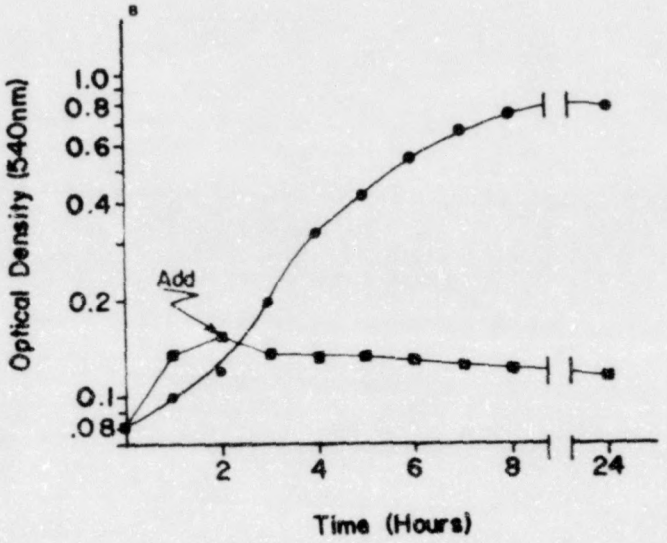
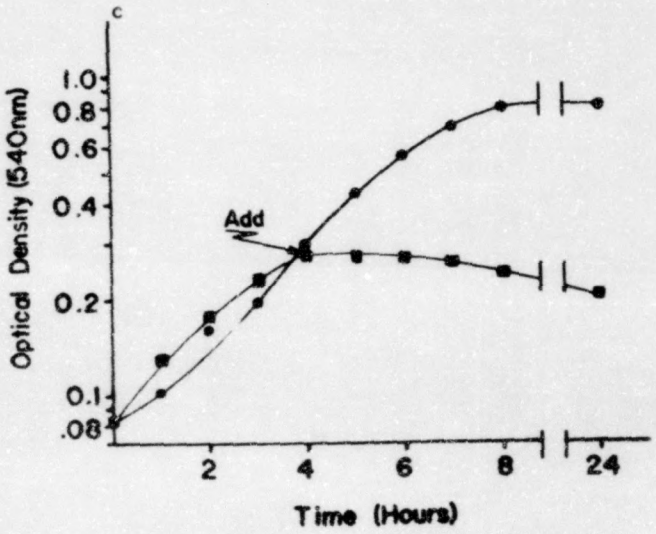
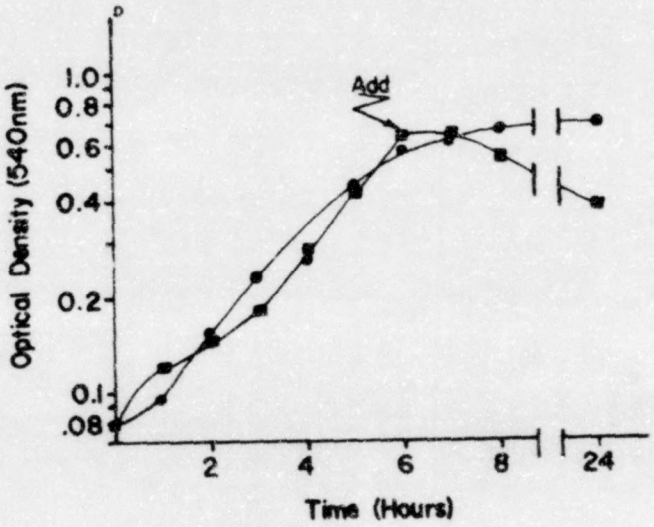

D
Optical Density (540nm)
1.0
0.8
0.6
0.4
2
4
6
8
24
Time (Hours)

Figure 7. Effect of time of addition of Everninomicin B induced inhibition of Bacillus subtilis W23. A: compound added at time 0, B: compound added after 2 hours incubation, C: compound added after 4 hours incubation, D: compound added after 6 hours incubation. (●) glucose minimal broth controls; (■) 6.5×10^{-3} µmole/ml of EvB in glucose minimal broth.


A
Optical Density (540nm)
1.0
0.8
0.6
0.4
0.2
0.1
.08
.06
Add
2
4
6
8
24
Time (Hours)


B
Optical Density (540nm)
1.0
0.8
0.6
0.4
0.2
0.1
.08
Add
2
4
6
8
24
Time (Hours)


C
Optical Density (540nm)
1.0
0.8
0.6
0.4
0.2
0.1
.08
Add
2
4
6
8
24
Time (Hours)


D
Optical Density (540nm)
1.0
0.8
0.6
0.4
0.2
0.1
.08
Add
2
4
6
8
24
Time (Hours)

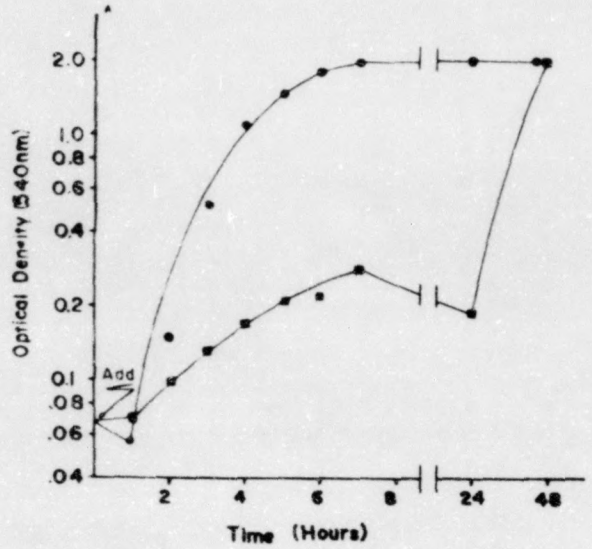
inhibition and no inhibition after 48 hours. When EvB was added to approximately two-hour actively growing cultures there was 33% inhibition of growth and no inhibition after 48 hours. When the compound was added to S. aureus which had been actively growing for 2½ hours there was 20% growth inhibition after 24 hours and no inhibition after 48 hours. These results indicated that for S. aureus the effect of EvB-induced growth inhibition was dependent upon the time of addition (Figure 8).

Viability Determinations

Viability determinations were used to determine if EvB-induced inhibition was bacteriostatic or bacteriocidal. B. subtilis W23 was grown in glucose minimal broth and plated on glucose minimal agar. The results showed that after a 90-minute incubation period there was no increase in colony forming units/ml (CFU/ml), which indicated that EvB was bacteriostatic for B. subtilis W23 (Figure 9).

Similar viability determinations as described previously were performed on the penicillin-resistant S. aureus except that this strain was grown in brain heart infusion broth and plated on brain heart infusion agar. After a 90-minute incubation period there was no increase in CFU/ml which indicated that for S. aureus the effect of EvB was bacteriostatic (Figure 10).

Figure 8. Effect of time of addition on Everninomicin B induced growth inhibition of penicillin-resistant Staphylococcus aureus. A: compound added at time 0, B: compound added after 1.0 hours, C: compound added after 2.25 hours, D: compound added after 2.5 hours. (●), brain heart infusion broth controls: (■) 6.5×10^{-3} µmole/ml of EvB in brain heart infusion broth.


Optical Density (540nm)
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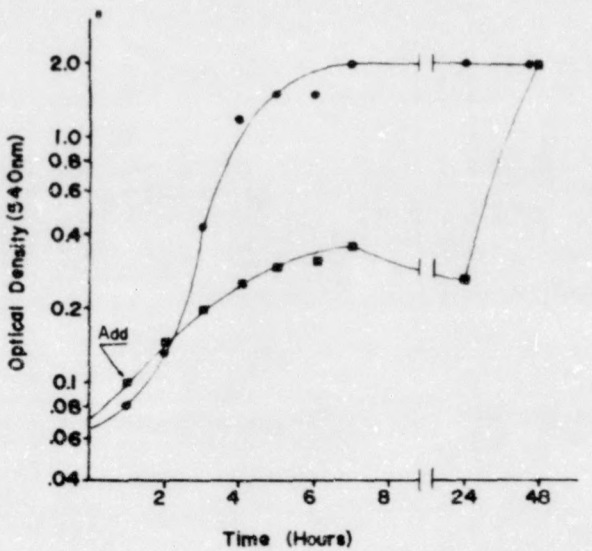
0.8
0.6
0.4
0.2
0.1
.08
.06
.04
Add
2
4
6
8
24
48
Time (Hours)


Optical Density (540nm)
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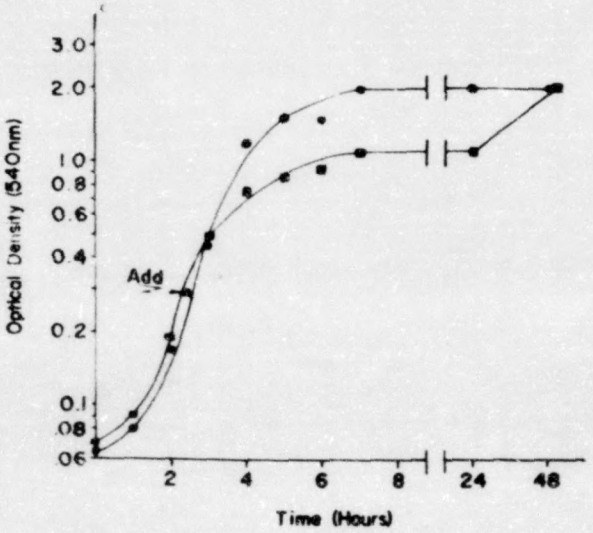
1.0
0.8
0.6
0.4
0.2
0.1
.08
.06
.04
Add
2
4
6
8
24
48
Time (Hours)


Optical Density (540nm)
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2.0
1.0
0.8
0.6
0.4
0.2
0.1
.08
.06
Add
2
4
6
8
24
48
Time (Hours)

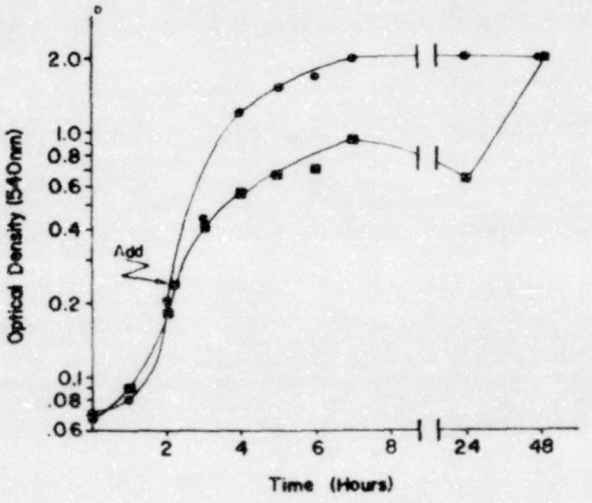

Optical Density (540nm)
2.0
1.0
0.8
0.6
0.4
0.2
0.1
.08
.06
Add
2
4
6
8
24
48
Time (Hours)

Figure 9. Effect of Everninomicin B on the viability of Bacillus subtilis W23. (●), glucose minimal medium controls; (■), 1.3×10^{-3} μmole/ml of EvB in glucose minimal broth.

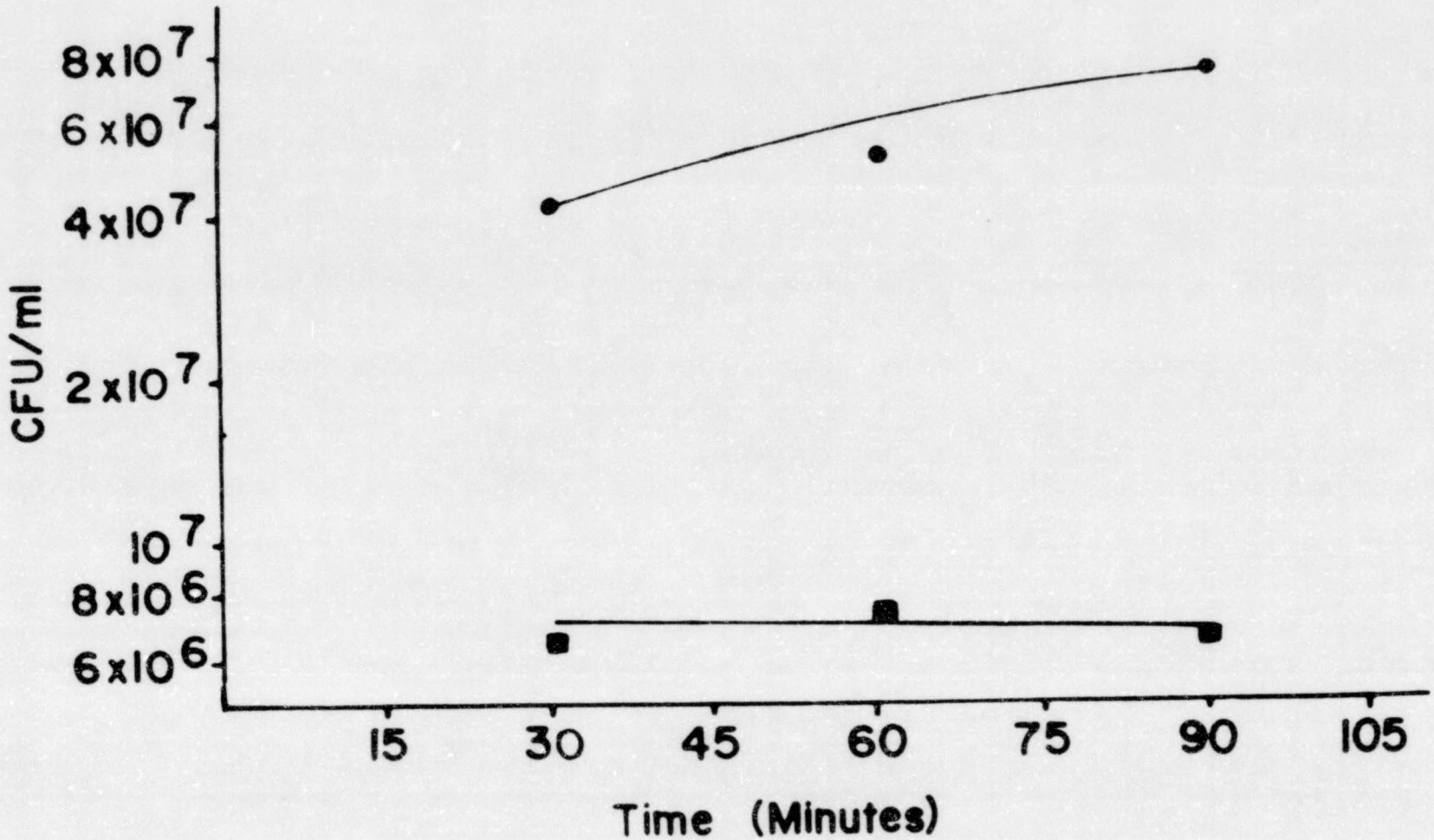
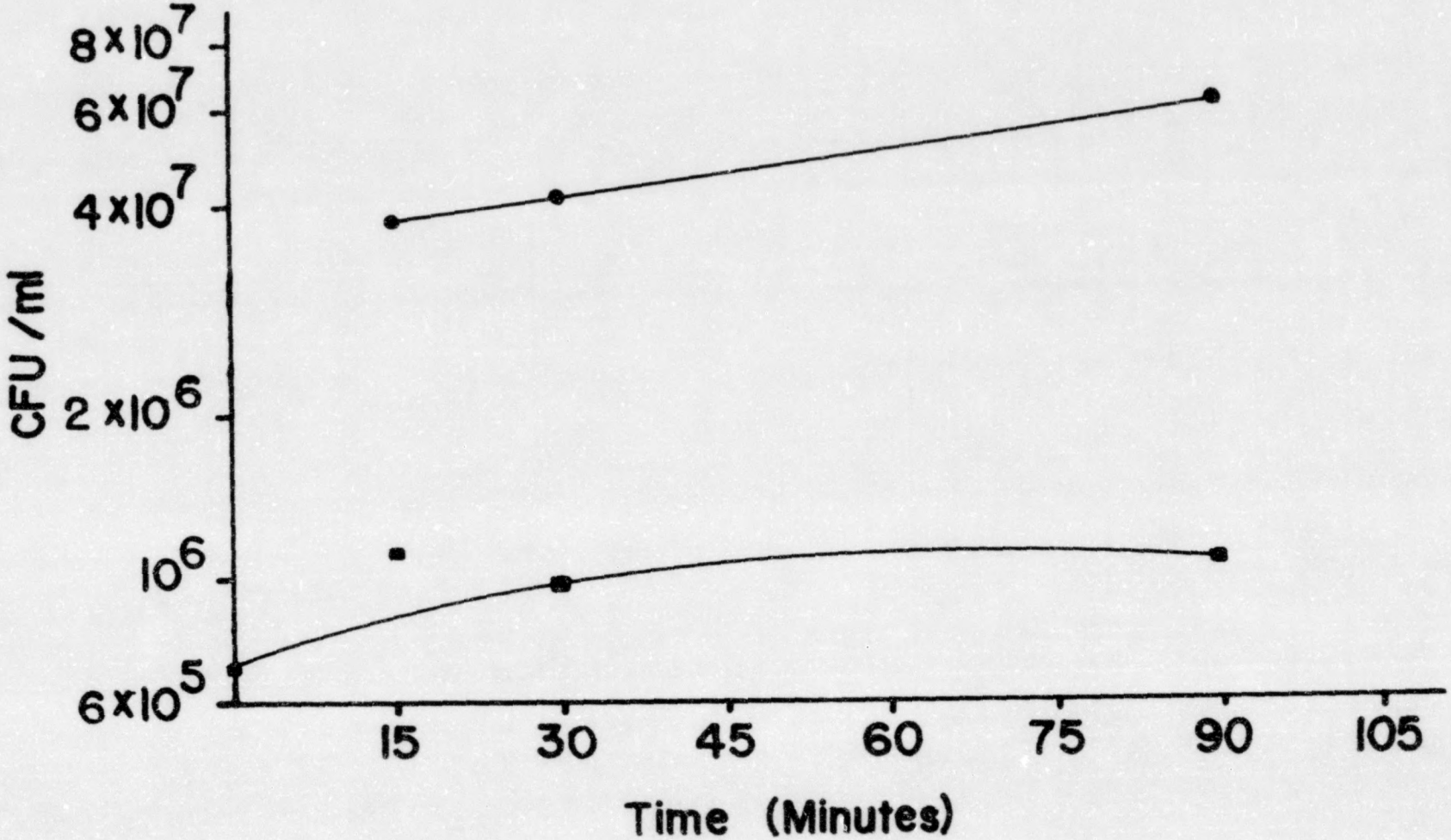

8x10^7
6x10^7
4x10^7
2x10^7
10^7
8x10^6
6x10^6
CFU/ml
15
30
45
60
75
90
105
Time (Minutes)

Figure 10. Effect of Everninomicin B on the viability of penicillin-resistant Staphylococcus aureus. (●), brain heart infusion medium controls; (■), 1.3×10^{-2} μmole/ml of EvB in brain heart infusion broth.


8x10^7
6x10^7
4x10^7
2x10^6
10^6
6x10^5
CFU/ml
15
30
45
60
75
90
105
Time (Minutes)

Electron Microscopy

When control cells of penicillin-resistant S. aureus were examined with an electron microscope, the cells had shown typical cluster formation and uniform morphology (Figure 11). When the penicillin-resistant S. aureus was treated with 1.3×10^{-3} µmole of EvB per ml and incubated for 18 hours on a rotary shaker at $36^{\circ}C$ it was noted that the treated cells showed marked pleomorphism, and lysis of some of the treated cells occurred (Figure 12).

Figure 11. Electron micrograph of untreated penicillin-resistant Staphylococcus aureus. Note regular shape of cells within the cluster.

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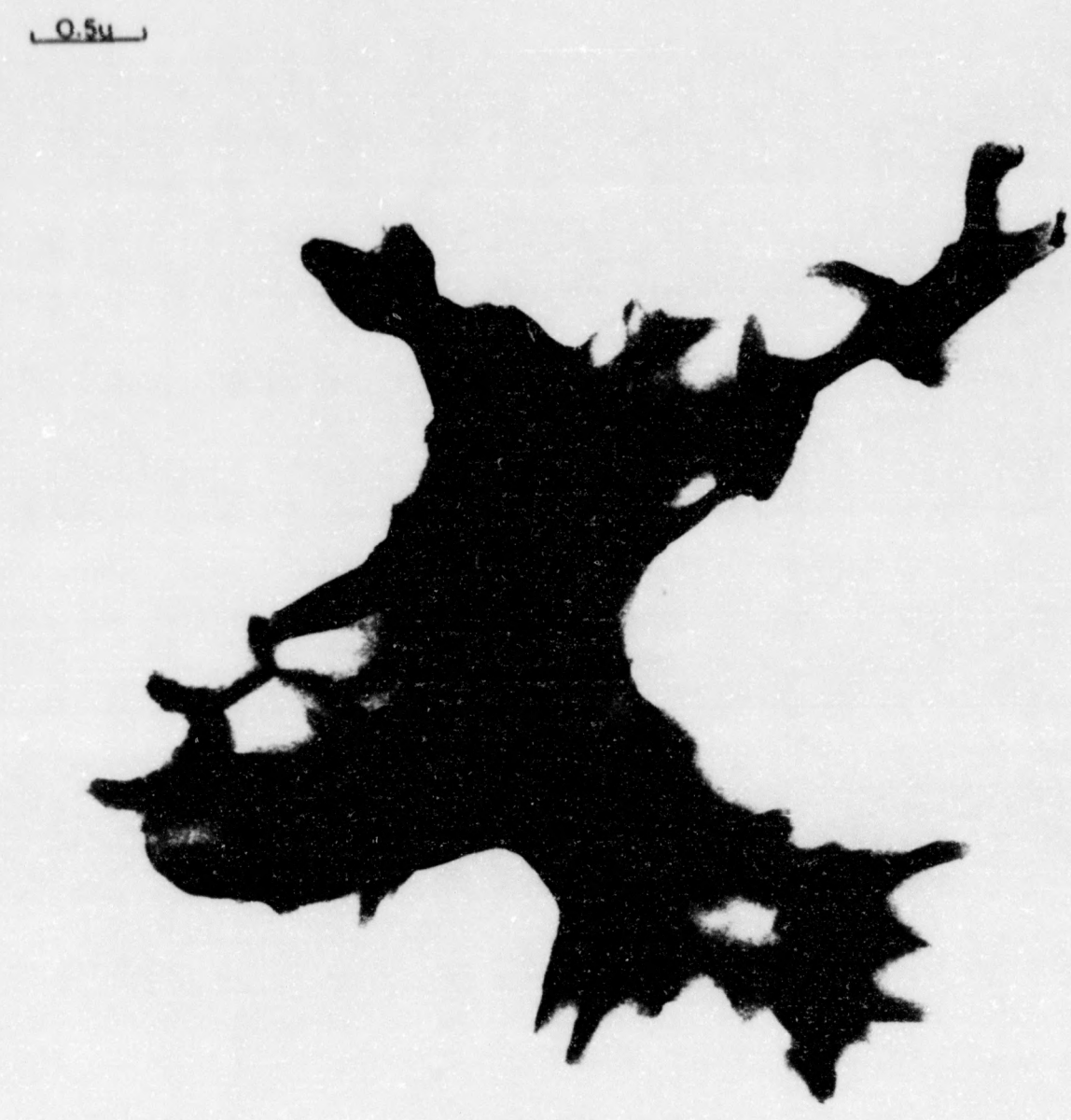
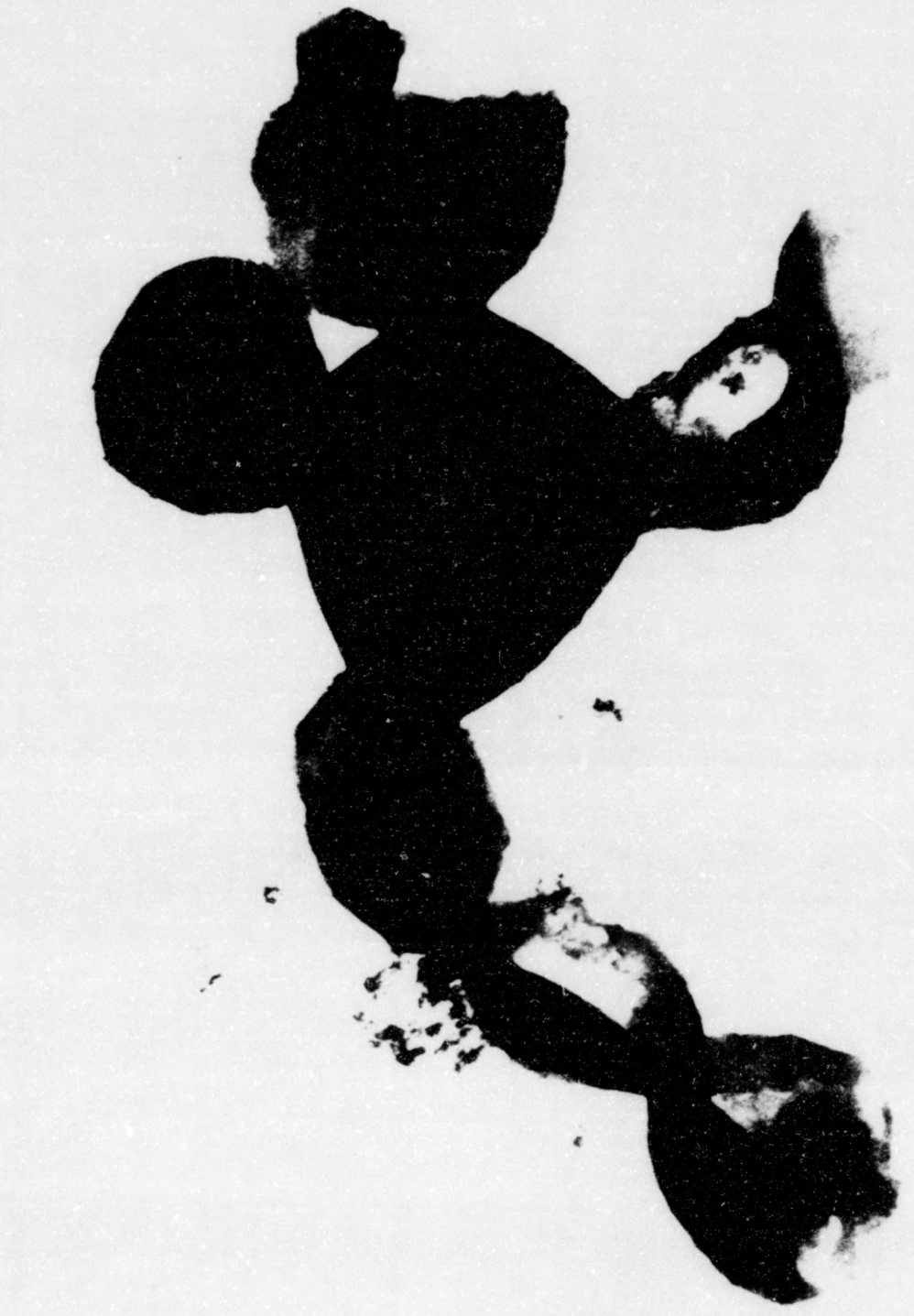

0.5μ

Figure 12. Electron micrograph of Everninomicin B treated penicillin-resistant Staphylococcus aureus. S. aureus was treated with 1.3×10^{-3} µmole/ml of EvB for 18 hours at 36°C. Note pleomorphic cells within the cluster and apparent lysis of some of these cells.


0.5u

DISCUSSION AND CONCLUSIONS

Micromonospora carbonacea (NRRL 2972) and M. carbonacea var. aurantiaca have been demonstrated to produce a complex of antibiotics known as the everninomicins (Wagman, et al., 1965). Of the five everninomicins complexes, EvD is produced in the greatest amount (Herzog, et al., 1965); because of this most of the pharmacological research has been done with EvD (Weinstein, et al., 1964; Black, et al., 1965). Herzog, et al. (1965) showed that EvD and EvB are structurally related. Although EvB has primarily a gram-positive spectrum (Black, et al., 1965), Sanders and Sanders (1978) showed that antibiotic-resistant bacteria are sensitive to EvB. They also demonstrated that there was no de novo resistance which suggested that EvB may have a novel mode of action. More research is needed to elucidate possible modes of action of EvB.

In this study the tube-dilution protocol showed that the bacterial strains tested were sensitive to the inhibitory effects of EvB. This was reflected by the fact that EvB had low MIC values for all bacterial strains tested.

When B. subtilis was grown in nutrient broth there was a two-fold increase in the MIC value. It was possible that the biological effects of EvB in nutrient broth were decreased as the natural complex of nutrients in the media increased

(Foster and Pittillo, 1952). The MIC values for the three antibiotic-resistant strains of S. aureus agree with the results of Sanders and Sanders (1978).

Tube-dilution reversibilities showed that the inhibitory effect was reversible for B. subtilis W23 with concentrations of EvB ranging from 1.3×10^{-3} to 1.05×10^{-2} μmole/ml when B. subtilis was grown in glucose minimal broth and 2.6×10^{-3} to 1.05×10^{-2} μmole/ml when B. subtilis was grown in nutrient broth. Tube-dilution reversiblities for three antibiotic-resistant strains of S. aureus showed that the inhibitory effect of EvB was reversible with concentrations ranging from 3.25×10^{-4} to 1.05×10^{-2} μmole/ml for the strains resistant to penicillin or tetracycline and 6.5×10^{-4} to 1.05×10^{-2} μmole/ml for S. aureus resistant to aminoglycosides.

In growth studies using B. subtilis W23 and S. aureus the concentration of EvB used was 6.5×10^{-3} μmole/ml which represented a five-fold increase in the MIC value for B. subtilis and a twenty-fold increase over the MIC value for S. aureus. At this concentration the inhibitory effect of EvB against B. subtilis W23 was not dependent upon the initial concentration of cells nor was it dependent upon when the compound was added to the actively growing cultures. The effect of EvB on penicillin-resistant S. aureus was dependent upon the initial concentration of cells as well as upon the time of addition of EvB. These results indicate that for S. aureus the higher

the concentration of cells (either actively growing or non-growing cells) the less inhibition of growth was detected.

Viability determinations for B. subtilis and S. aureus showed that after a 90-minute incubation period there was no increase in CFU/ml in the treated cultures. The results of the viability determinations and reversibility studies indicated that EvB was bacteriostatic.

A probable explanation for the differences between the results of B. subtilis and S. aureus was the difference in the media. B. subtilis, often used in antibiotic research, can be grown in minimal media. S. aureus can only be grown in complex media which could reduce the inhibitory effect of the compound on bacterial growth (Foster and Pittillo, 1952).

Electron microscopy showed that when cells of S. aureus were exposed to EvB and compared to untreated cells there were distinct morphological changes in the untreated cells. These changes included marked pleomorphism and lysis. It could not be determined from this study if the lysis of the treated cells was due to the actual effect of the compound or if cellular lysis was due to the physical effects of the vacuum when the cells were placed into the electron microscope.

In evaluating EvB for possible use as a clinically significant antibiotic, more research is needed to elucidate its exact mode of action, a suitable vehicle for injection must be found, and an analogue which will not bind readily to human serum should be synthesized. Further alternative

research should include the possible use of EvB in selective media, or as a preservative of foodstuffs because of its low host toxicity.

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