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FOR BACILLUS ANTHRACIS

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U.S. ARMY BIOLOGICAL CENTER
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TECHNICAL MANUSCRIPT 284

A SELECTIVE MEDIUM FOR BACILLUS ANTHRACIS

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COMMODITY DEVELOPMENT AND ENGINEERING LABORATORY

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ABSTRACT

A selective medium that allows for growth of Bacillus anthracis while inhibiting common contaminants and closely related spore-formers (e.g., Bacillus cereus) is described. This medium contains polymyxin, lysozyme, disodium ethylenediamine tetraacetate, and thallous acetate as the selective ingredients. It may also be used as a differential medium to distinguish B. anthracis from B. cereus and may be of value in the classification of Bacillus species.

I. INTRODUCTION

A need has existed for a selective medium that allows for growth of Bacillus anthracis while inhibiting common contaminants and closely related sporeformers (e.g., Bacillus cereus). The selective media developed by Pearce and Powell,¹ Morris,² and Gillissen and Scholz³ do not inhibit the growth of B. cereus. A new selective medium containing polymyxin, lysozyme, disodium ethylenediamine tetraacetate (EDTA), and thallous acetate is described in the present study. This medium will inhibit the growth of the majority of B. cereus strains while permitting growth of both virulent and avirulent strains of B. anthracis. It can also be used as a differential medium for B. anthracis and should be of value when employed with two other differential media previously described.⁴ These previously described media inhibited the growth of B. anthracis while permitting recovery of B. cereus. When it is used as a differential medium, polymyxin may be eliminated as one of the ingredients.

II. MATERIALS AND METHODS

A. STRAINS

The strains tested included 21 B. anthracis, 11 B. cereus, 5 B. cereus var. mycoides, 23 miscellaneous Bacillus species, 3 Escherichia coli, and one each of Aerobacter aerogenes, Alcaligenes faecalis, Proteus mirabilis, Proteus vulgaris, Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus faecalis, and Paracolobacterium sp. (Table 1). One spore suspension of B. anthracis was also tested. All B. anthracis strains were lysed by gamma phage.⁵

B. PREPARATION OF CELLS

Growth from 24-hr heart infusion agar (HIA) (Difco) slants was removed and suspended in 10 ml of 0.06 M phosphate buffer (pH 7.3). Triplicate plates of the test media were inoculated with 0.1 ml of an appropriate dilution and were incubated at 37 C for 24 to 48 hours.

C. PREPARATION OF THE MEDIUM

Polymyxin (Burroughs Wellcome Co., Tuckahoe, N.Y.) was resuspended in distilled water. Lysozyme (Armour, Kankakee, Ill.), EDTA, and thallous acetate (Fisher Scientific Co., Fairlawn, N.J.) were dissolved in distilled water and sterilized by Seitz-filtration. The basal HIA (Difco) was sterilized and cooled to 45 to 50 C and the following ingredients were added: polymyxin, 50 units/ml; lysozyme, 40 µg/ml; EDTA, 300 µg/ml; and thallous

TABLE 1. RECOVERY OF BACILLUS SPECIES ON THE SELECTIVE MEDIUM

Organism	24-Hr Recovery, 7a/	Organism	24-Hr Recovery, 7a/
<u>B. anthracis</u>		<u>B. megaterium</u>	
12	80	ATCC 11561, ATCC 11561-E,	
ATCC 944	None ^{b/}	ATCC 8245	None
15	135		
Sax	87	ATCC 6458	162
Vollum B	133		
Avir Vaccine str.	80	<u>B. polymyxa</u>	
Michigan Vollum	71		
Ohio	64	ATCC 8526	None
1014	75		
V1b	110	<u>B. pumilus</u>	
V1b (spores)	80		
Willard	88	ATCC 7061, ATCC 4510	None
HBA 199	78		
HBA 201	71	<u>B. sphaericus</u>	
HBA 273	104		
D-379	78	X-2, ATCC 4525, ATCC 248	None
D-380	42		
D-382	42	<u>B. sphaericus</u> var. <u>fusiformis</u>	
D-384	80		
D-390	53	ATCC 7054	None
D-417	57		
HBA 87	100	<u>B. subtilis</u>	
<u>B. cereus</u>		ATCC 9466, ATCC 9524,	
ATCC 4507, ATCC 7004,		ATCC 9860, ATCC 8480,	
V6, ATCC 7483, ATCC 9620,		ATCC 6051	None
V7, ATCC 9139, ATCC 6464,			
HBA 112, ^{c/} HBA 248 ^{c/}	None	<u>B. subtilis</u> var. <u>niger</u>	
ATCC 7064	164	ATCC 6455, ATCC 7972	None
		ATCC 6537	74
		ATCC 9372	10
		BG III	100
<u>B. cereus</u> var. <u>mycoides</u>			
ATCC 6462, ATCC 6463,		<u>B. thuringiensis</u>	
NRS 306, NRS 327,			
ATCC 10206	None	NRS 1328, ATCC 10792	None

a. Recovery compared with HIA (as 100% recovery control).

b. Recovery of 29% was obtained after 48-hr incubation.

c. Strains originally classified as B. anthracis - See Results.

acetate, 40 µg/ml (PLET medium). The final unadjusted pH of the medium was 7.35. For comparison, the PLET medium was also prepared with heart infusion broth (HIB) (Difco) containing 1.5% of added agar (Difco) as well as another source of heart infusion agar (Fisher). Plain HIA (Difco) was used as the regular 100% recovery control medium. HIB (Difco) and HIA (Fisher) were also employed as 100% recovery controls when the PLET medium was prepared in these basal media.

III. RESULTS

A. RECOVERY OF B. ANTHRACIS STRAINS ON THE SELECTIVE MEDIUM

The recovery of 21 strains of B. anthracis is shown in Table 1. Good recovery was obtained in 24 hours (37 C) with all strains except one (ATCC 944). This strain required 48 hours to yield a recovery of 29%. A spore suspension of strain V1b was recovered without difficulty. Typical recovery obtained with one B. anthracis strain is shown in Figure 1. Colonies of B. anthracis are smaller and smoother on the selective medium than on the control medium.

B. INHIBITION OF B. CEREUS AND B. CEREUS VAR. MYCOIDES ON THE SELECTIVE MEDIUM

All of the 11 strains tested were inhibited except ATCC 7064 (Table 1). On the selective medium, the colonies of this strain were very minute at the end of 24 hours (37 C) incubation, and after 48 hours, the colonies were small and approximately the size of 24-hr B. anthracis colonies. Two strains (HBA 112 and HBA 248), originally classified as B. anthracis and subsequently identified as B. cereus,⁴ were completely inhibited on the PLET medium, thus supplying additional evidence to justify their classification. Figure 2 shows typical inhibition of a B. cereus strain after 48-hours incubation.

C. INHIBITION OF MISCELLANEOUS BACILLUS SPECIES

The majority of other Bacillus sp. were also inhibited on the PLET medium with the exception of one "lysozyme-resistant" Bacillus megaterium strain (ATCC 6458) and several of the Bacillus subtilis strains. The results are shown in Table 1.

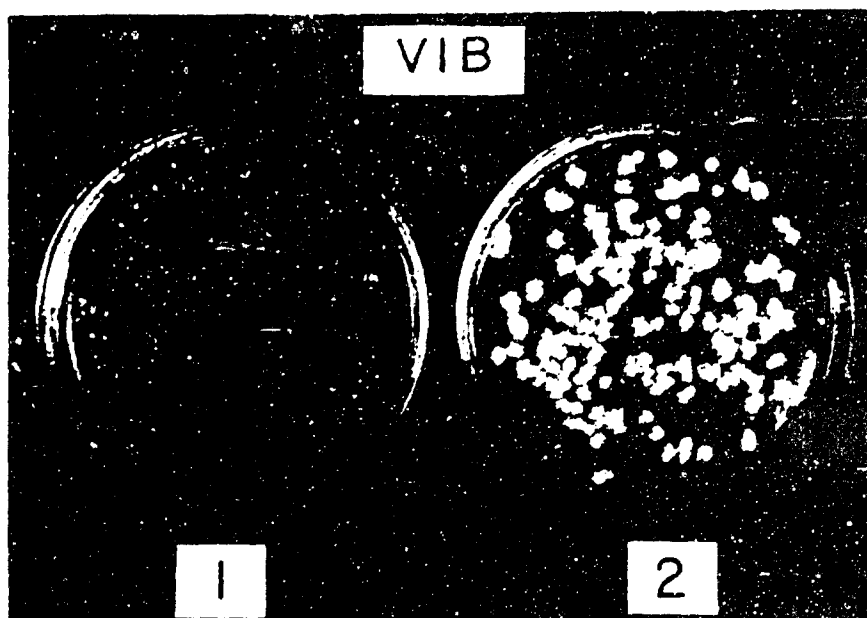


Figure 1. Typical Recovery of One Strain of B. anthracis after 24-Hours Incubation at 37 C on (1) Selective Medium and (2) HIA Control.

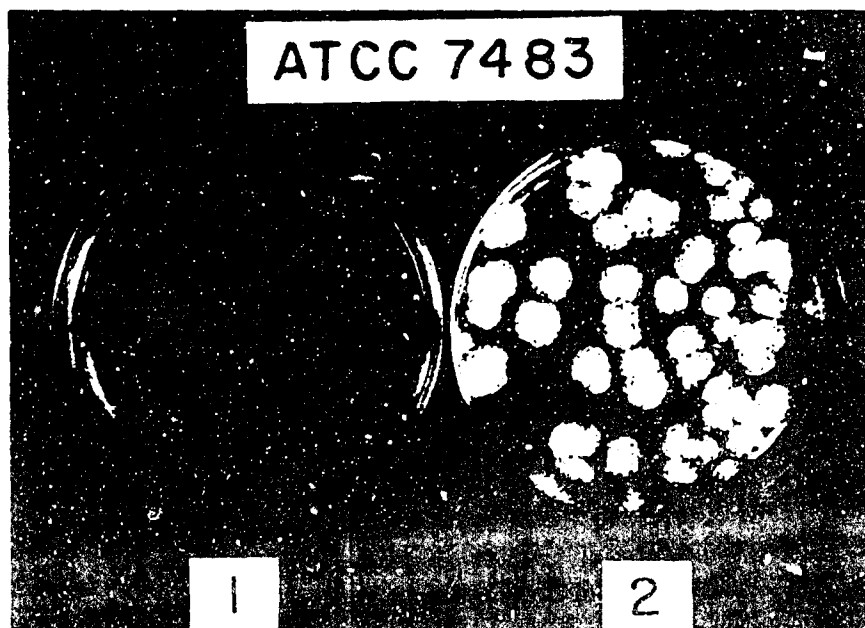


Figure 2. Typical Inhibition of One Strain of B. cereus after 48-Hours Incubation at 37 C on (1) Selective Medium Compared with (2) the HIA Control.

D. INHIBITION OF OTHER ORGANISMS

Aerobacter aerogenes, Alcaligenes faecalis, Escherichia coli, Escherichia intermedium, Paracolobactrum sp. and Pseudomonas aeruginosa were completely inhibited. The spreading of Proteus vulgaris was prevented for 24 hours, although colonies did appear. Spreading occurred upon further incubation. Staphylococcus aureus and Streptococcus faecalis were not inhibited.

E. RECOVERY WITH OTHER BASAL MEDIA

Recovery of B. anthracis strains was generally less on a selective medium prepared with HIB (Difco) and 1.5% agar (as the basal medium) than on the selective medium prepared with different lot numbers of HIA (Difco). No recovery of B. anthracis strain ATCC 944 was obtained with the former medium even after 48-hours incubation and the recovery of B. cereus strain ATCC 7064 was decreased to 63%, indicating greater inhibition with this medium. Higher recovery of most B. anthracis strains was obtained with a selective medium prepared in HIA (Fisher) basal medium compared with recovery on the selective medium prepared in HIA (Difco). Unfortunately, some recovery of B. cereus strains also occurred.

IV. DISCUSSION

Gillissen and Scholz³ reported the value of polymyxin for inhibition of gram-negative organisms when isolating B. anthracis. Pearce and Powell¹ prepared a selective medium for B. anthracis containing hematin and lysozyme. This medium was unsatisfactory because of the inhibitory action of freshly prepared hematin on the recovery of many gram-positive organisms.⁸ The lysozyme was of value in inhibiting certain sporeforming bacilli (other than B. anthracis), and therefore was retained in the present medium.

Thallous acetate has been used frequently to control the spreading of bacterial colonies and also to inhibit gram-negative organisms.^{7,8} Thallous acetate controlled the spreading of B. cereus var. mycoides and inhibited some other organisms when added to the polymyxin-lysozyme medium.

The addition of EDTA to this medium resulted in the unique action whereby B. anthracis strains were easily recovered while B. cereus strains were generally inhibited. This phenomenon occurred only when the thallous acetate and EDTA were combined; separately they did not exhibit this

action. The specific interactions that may be involved are not known at this time. It is possible that EDTA (as a chelating agent) combines with an essential cation⁹ required by these organisms and that thallium may be utilized as a substitute by B. anthracis but not by B. cereus. Further investigation of this phenomenon is now being conducted.

The concentration of ingredients in the PLET medium was effective only in a basal medium of HIA (Difco). If other basal media are used these concentrations will probably require some modification. Adjustment of the pH is generally not recommended, regardless of the basal medium used.

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