Microbial Ecology of *Bacillus thuringiensis* V. Selective Medium for *Bacillus thuringiensis* Vegetative Cells

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Bacillus thuringiensis vegetative cells were capable of growing on a defined media containing acetate or citrate. In contrast, no growth of this bacterium was detected on these media when spores were used as the inocula. Based on the assimilation tests of organic compounds, a selective medium for vegetative cells (BTV medium) was devised for *B. thuringiensis* subsp. *thuringiensis* and subsp. *morrisoni*. Composition of the medium was as follows: NaNO₃, 1.0g; KH₂PO₄, 1.0 g; MgSO₄·7H₂O, 0.5 g; KCl, 0.2 g; sodium acetate, 1.0 g; sodium citrate, 3.0 g; Difco purified agar, 15.0 g; polymyxin B, 5 mg; deionized water, 1,000 ml; pH 7.0.

INTRODUCTION

In the course of ecological studies on *Bacillus thuringiensis*, we found that the number of viable *B. thuringiensis* cells in soils treated with this bacterium gradually decreased owing to the existence of competitive microorganisms and low soil pH (SEKIJIMA et al., 1977; AKIBA et al., 1977, 1979, 1980). In these studies, the number of viable cells in a mixture of vegetative cells and spores was counted on a semiselective nutrient agar medium (SALEH et al., 1970). In order to estimate the number of *B. thuringiensis* spores, the sample was heated at 80°C for 10 min. The number of viable vegetative cells was estimated by excluding the number of viable spores from the total number in the mixture. However, this technique is not applicable for the small number of *B. thuringiensis* vegetative cells in the soil. To overcome problems in the usual plating technique, the use of immunofluorescence in the detection of vegetative cells in soil was reported by WEST et al. (1984).

In the present paper, we report a new selective medium based on the assimilation of carbon sources for the detection of a small number of *B. thuringiensis* vegetative cells in the soil.

MATERIALS AND METHODS

Bacteria. Twenty-one type strains of *B. thuringiensis* (Table 1) used in this study were supplied by Dr. K. AIZAWA, Kyushu University. A strain of *Bacillus cereus* isolated from the soil (KATO and SUZUKI, 1979) was also used in the experiment.

Bacteria were cultured at 30°C for 24-48 hr on nutrient agar medium and harvested in distilled water. To prepare a spore suspension, cells were heated at 80°C for 10 min.

Assimilation tests. A mixture of vegetative cells and spores and heat resistant cells

(spores) were used as the inocula for the assimilation tests. Growth of bacteria was examined at 30°C for 7 days on the basal medium [KH_2PO_4 , 1 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g; KCl, 0.2 g; $(\text{NH}_4)_2\text{SO}_4$, 0.8 g; Difco purified agar, 15 g; deionized water, 1,000 ml; pH 7.0] supplemented with each of organic compounds (Table 1) at a concentration of 2 g/ 1,000 ml.

Nitrogen and carbon sources for selective medium of B. thuringiensis vegetative cells. Growth of two type strains of *B. thuringiensis*, subsp. *thuringiensis* and subsp. *morrisoni*, was examined on fifteen defined media consisting of the above basal medium and various combinations of nitrogen and carbon sources (Table 2). Polymyxin B was added to these media at a concentration of 5 ppm. Mixtures of vegetative cells and spores or spores alone were inoculated on the plates of these media, and after incubation at 30°C for 5 days, the number of colonies formed was counted.

Comparison of two techniques for vegetative cell counts. The count of viable vegetative cells of *B. thuringiensis* subsp. *thuringiensis* and subsp. *morrisoni* was performed on the plates of a newly defined medium developed on the basis of assimilation tests. The numbers of vegetative cells of the two strains were also estimated by excluding the number of heat resistant spores from the total number in the mixture of vegetative cells and spores. For this count, peptone-polymyxin medium (PP medium: peptone, 10 g; NaCl, 5 g; agar, 15 g; polymyxin B, 5 mg; distilled water, 1,000 ml; pH 8.5) was used.

Direct counting of the actual number of vegetative cells and spores of these two strains was done using a microscope with a counting chamber (Erma, Tokyo).

RESULTS AND DISCUSSION

Assimilation of organic compounds

The results of assimilation tests of *B. thuringiensis* and *B. cereus* strains are shown in Table 1. When mixtures of vegetative cells and spores of *B. thuringiensis* (subsp. *thuringiensis* and subsp. *morrisoni*) were used as the inocula, bacterial growth was evident on the medium containing acetate or citrate. In contrast, there was no growth of these two strains on the above two media when spores were tested as the inocula.

These results indicate that only vegetative cells of these two strains were able to grow on the medium containing citrate or acetate as a carbon source, suggesting that spores did not germinate on these media. Similar results were obtained in most strains of *B. thuringiensis* and a *B. cereus* strain.

The data also suggest that most strains of *B. thuringiensis* do not require any amino acids. This observation supports the result reported by NICKERSON and BULLA (1974).

Based on the assimilation tests, a further test for selective medium was performed using combinations of the type strains of subsp. *thuringiensis* and subsp. *morrisoni* and media containing acetate and citrate.

Comparative cell numbers on defined media

Fifteen defined media were tested for their ability to support the growth of the type strains of subsp. *thuringiensis* and subsp. *morrisoni*. As shown in Table 2, the largest number of colonies of both strains was obtained on the No. 5 medium, when mixtures of spores and vegetative cells were inoculated. On the contrary, no colony formation was observed on this medium when spores were used as the inocula. On this medium, colonies were formed after incubation at 30°C for 5 days and the colony morphologies

Table 1. Assimilation of organic compounds by *Bacillus thuringiensis* and *Bacillus cereus*

Bacteria	Inoculum ^a	Organic compounds															
		D-glucose	D-fructose	Sucrose	Trehalose	Maltose	Cellobiose	Starch	Gluconate	Acetate	Citrate	Glycerol	L-glutamate	DL-arginine	L-ornithine	L-histidine	Aspartate
<i>Bacillus thuringiensis</i>																	
subsp. <i>thuringiensis</i>	M	++ ^b	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	S	+	—	—	—	—	—	—	+	—	—	—	+	+	+	—	+
<i>finitimus</i>	M	— ^c	—	—	—	—	—	—	—	+	+	—	+	—	+	+	+
	S	—	—	—	—	—	—	—	—	—	—	—	+	+	+	+	+
<i>alesti</i>	M	—	—	—	—	—	—	—	—	+	+	—	+	+	+	+	+
	S	—	—	—	—	—	—	—	—	—	—	—	+	+	+	+	+
<i>kurstaki</i>	M	—	—	—	—	—	—	—	—	+	+	—	+	—	+	+	+
	S	—	—	—	—	—	—	—	—	—	—	—	+	—	+	—	—
<i>sotto</i>	M	—	—	—	—	—	—	—	—	—	+	—	+	—	—	+	—
	S	—	—	—	—	—	—	—	—	—	—	—	+	—	+	—	—
<i>dendrolimus</i>	M	—	+	—	+	+	+	+	—	+	—	+	+	—	—	—	+
	S	—	—	—	—	—	—	—	—	—	—	—	+	—	—	—	+
<i>kenyae</i>	M	+	—	+	+	—	+	+	—	+	+	+	+	+	+	+	+
	S	—	—	—	—	—	—	—	—	+	—	+	+	—	—	—	+
<i>galleriae</i>	M	+	+	+	+	+	+	+	+	+	—	+	+	+	+	+	+
	S	—	—	—	—	—	—	—	—	—	—	—	+	—	+	—	—
<i>canadensis</i>	M	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	S	—	—	—	—	—	—	—	—	—	—	—	+	—	+	—	+
<i>entomocidus</i>	M	—	—	—	—	—	—	—	—	—	+	—	+	+	+	+	+
	S	—	—	—	—	—	—	—	—	—	—	—	+	—	+	—	+
<i>subtoxicus</i>	M	—	—	—	—	—	—	—	—	—	+	—	+	+	+	—	+
	S	—	—	—	—	—	—	—	—	—	—	—	+	—	+	—	—
<i>aizawai</i>	M	—	—	—	—	—	—	—	—	—	—	—	+	—	—	—	—
	S	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>morrisoni</i>	M	—	—	—	—	—	—	—	—	+	+	—	+	+	+	+	+
	S	—	—	—	—	—	—	—	—	—	—	—	+	—	+	—	—
<i>ostrinae</i>	M	—	—	—	—	—	—	—	—	—	+	—	+	+	+	—	+
	S	—	—	—	—	—	—	—	—	—	—	—	+	—	+	—	+
<i>tolworthi</i>	M	—	+	—	—	—	—	—	—	+	+	—	+	+	+	+	—
	S	—	—	—	—	—	—	—	—	—	—	—	+	—	+	—	—
<i>darmstadiensis</i>	M	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	S	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>toumanoffii</i>	M	—	—	—	—	—	—	—	—	+	+	—	+	+	+	+	+
	S	—	—	—	—	—	—	—	—	—	—	—	+	—	+	—	+
<i>kyushuensis</i>	M	—	—	—	—	—	—	—	—	+	+	—	+	+	—	+	+
	S	—	—	—	—	—	—	—	—	—	—	—	+	—	—	—	+
<i>thompsoni</i>	M	—	—	—	—	—	—	—	—	+	+	—	+	+	+	+	+
	S	—	—	—	—	—	—	—	—	—	—	—	+	—	—	—	+
<i>pakistani</i>	M	+	+	+	+	+	+	—	—	+	+	—	+	+	+	—	+
	S	—	—	—	—	—	—	—	—	—	—	—	+	—	+	—	+
<i>israelensis</i>	M	+	+	—	+	+	+	—	+	+	+	—	+	+	—	—	+
	S	—	—	—	—	—	—	—	—	—	—	—	+	—	+	+	+
<i>Bacillus cereus</i>	M	—	—	—	—	—	—	—	—	+	+	+	+	+	+	+	+
	S	—	—	—	—	—	—	—	—	—	—	—	+	—	+	—	—

^a Inoculum: M, mixture of vegetative cells and spores; S, spores.^b Bacterial growth was observed.^c Bacterial growth was not observed.

Table 2. Nitrogen and carbon sources for selective medium of *Bacillus thuringiensis* vegetative cells

15 defined media ^a			Inoculum ^d			
Medium No.	Nitrogen ^b source	Carbon ^c source	subsp. <i>thuringiensis</i>		subsp. <i>morrisoni</i>	
			M	S	M	S
1	NaNO ₃	C	2.5 × 10 ^{5e}	0	2.0 × 10 ⁶	0
2	NaNO ₃	C+G	4.0 × 10 ⁵	0	8.5 × 10 ⁵	0
3	NaNO ₃	C+S	1.5 × 10 ⁵	0	5.0 × 10 ⁵	0
4	NaNO ₃	C+M	3.5 × 10 ⁵	5.0 × 10	1.0 × 10 ⁶	0
5 ^f	NaNO ₃	C+A	9.5 × 10 ⁵	0	3.3 × 10 ⁶	0
6	NH ₄ NO ₃	C	3.5 × 10 ⁵	0	6.0 × 10 ⁵	0
7	NH ₄ NO ₃	C+G	5.5 × 10 ⁵	0	3.5 × 10 ⁵	1.0 × 10 ²
8	NH ₄ NO ₃	C+S	8.5 × 10 ⁵	0	5.5 × 10 ⁵	5.0 × 10
9	NH ₄ NO ₃	C+M	4.5 × 10 ⁵	0	6.0 × 10 ⁵	0
10	NH ₄ NO ₃	C+A	7.0 × 10 ⁵	0	8.0 × 10 ⁵	0
11	(NH ₄) ₂ SO ₄	C	4.5 × 10 ⁵	0	6.5 × 10 ⁵	0
12	(NH ₄) ₂ SO ₄	C+G	4.5 × 10 ⁵	0	1.2 × 10 ⁶	0
13	(NH ₄) ₂ SO ₄	C+S	9.5 × 10 ⁵	5.0 × 10	9.5 × 10 ⁵	0
14	(NH ₄) ₂ SO ₄	C+M	5.0 × 10 ⁵	0	1.7 × 10 ⁶	5.0 × 10
15	(NH ₄) ₂ SO ₄	C+A	1.0 × 10 ⁶	0	1.4 × 10 ⁶	0

^a Nitrogen and carbon sources were added to the basal medium: KH₂PO₄, 1 g; MgSO₄·7H₂O, 0.5 g; KCl, 0.2 g; Difco purified agar, 15 g; polymyxin B, 5 mg; deionized water, 1,000 ml; pH, 7.0.

^b Concentration: 1 g/1,000 ml.

^c Carbon source: C, citrate; G, glucose; S, sucrose; M, maltose; A, acetate. Concentration: citrate, 3 g/1,000 ml; other carbon sources, 1 g/1,000 ml.

^d Inoculum: M, mixture of vegetative cells and spores; S, spores.

^e Number of colonies formed per milliliter.

^f Designated BTV medium.

Fig. 1. Colonies of *Bacillus thuringiensis* on BTV medium (×40).

A: *B. thuringiensis* subsp. *thuringiensis*. B: *B. thuringiensis* subsp. *morrisoni*.

of these two strains were apparently different from those of the other soil bacteria (Fig. 1).

From the above results, the No. 5 medium, designated BTV medium, was devised as the selective medium for vegetative cells of subsp. *thuringiensis* and subsp. *morrisoni*.

Table 3. Cell numbers obtained by three different methods

Method	Medium ^a	Inoculum ^b	Number of cells/ml	
			<i>B. thuringiensis</i> subsp. <i>thuringiensis</i>	<i>B. thuringiensis</i> subsp. <i>morrisoni</i>
Colony count	PP	M	2.0×10^6	13×10^6
		S	0.9×10^6	10×10^6
		V ^c	1.1×10^6	3×10^6
	BTV	V	2.2×10^6	3.5×10^6
		S	0	0
Direct microscopic count		V	7.0×10^6	13×10^6
		S	4.0×10^6	43×10^6

^a Medium: PP, peptone-polymyxin medium; BTV, selective medium for *Bacillus thuringiensis* vegetative cells.

^b Inoculum: M, mixture of vegetative cells and spores; V, vegetative cells; S, spores.

^c Number of vegetative cells was estimated by excluding the number of viable spores from the number of viable cells in the mixture.

Composition of the BTV medium was as follows: NaNO_3 , 1.0 g; KH_2PO_4 , 1.0 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g; KCl, 0.2 g; sodium acetate, 1.0 g; sodium citrate, 3.0 g; Difco purified agar, 15.0 g; polymyxin B, 5 mg; deionized water, 1,000 ml; pH 7.0.

Comparison of two media and a direct technique for vegetative cell numbers

As shown in Table 3, there was no difference in the numbers of vegetative cells counted on PP medium and BTV medium when a mixture of vegetative cells and spores was used as the inocula. In contrast, no colony formation was observed on BTV medium when spores were used as the inocula. This indicates that BTV medium can be used as a selective medium for vegetative cells of subsp. *thuringiensis* and subsp. *morrisoni*.

The number of vegetative cells and spores estimated by colony formation was less than the number obtained by a direct microscopic count, suggesting that dead cells were included in the physical count.

The BTV medium newly defined in this study will be useful for the ecological study of *B. thuringiensis* in soils and other environments.

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