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Enumeration and Identification of *Bacillus cereus* in Foods

I. 24-Hour Presumptive Test Medium

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An egg yolk-polymyxin medium (KG) for rapid enumeration of *Bacillus cereus* is described. The test is presumptive in that differentiation of *B. cereus* (and closely related organisms) from other species is based on the formation of turbidity in the agar surrounding the colonies of the *cereus* group organisms. The medium is formulated to encourage sporulation and release of free spores for serological confirmatory tests within the 24-hr incubation period. The production of turbidity in egg yolk and free-spore production by 25 strains of *B. cereus* on KG agar were measured. The recovery of food poisoning strains of *B. cereus* inoculated into nonsterile food slurries was assessed. A comparison of KG agar and mannitol-egg yolk-polymyxin-agar indicated that the two media were comparable in their abilities to recover low levels of *B. cereus* from naturally contaminated foods. Since KG agar enhances spore formation by *B. cereus*, thus permitting early serological testing, its use in screening food products is advocated.

The role of *Bacillus cereus* as an etiological agent of foodborne disease was described by Hauge (7, 8). Since then, other investigators have reported outbreaks and frequencies of *B. cereus* food poisoning (1, 11, 14, 15). Oddly, until 1970, there was not a single report of a well documented outbreak of *B. cereus* poisoning in the United States. To date, there have been no outbreaks of this illness reported in England (B. Hobbs, *personal communication*). In light of the rather high frequency of occurrence in Hungary (14), for example, it is quite probable that the infrequency of outbreaks in England and the United States reflects a lack of attention or examination of suspect foods rather than absence of occurrence. One possible explanation for this is that until recently a selective medium for *B. cereus* had not been described.

At present, most media employed for enumeration of *B. cereus* contain egg yolk. Thus, the primary differentiation of *B. cereus* from other *Bacillus* species depends on the detection of phospholipase C activity in cultures of the former organism. This stems from the early work of Colmer (4) and Chu (2), who described the presence of lecithinase in cultures of *B. cereus*. Donovan (5) devised a peptone-beef extract—2.5% egg yolk medium which also contained polymyxin-lithium chloride for the inhibition of gram-positive and gram-negative bacteria, respectively, and trisodium citrate to facilitate assessment of the egg yolk reaction.

More recently, Mossel et al. (12) described a similar medium (MYP) which omitted the lithium chloride and added D-mannitol and phenol red for differentiation purposes. These authors successfully employed this medium for the detection of *B. cereus* in various food products. They suggested the employment of further biochemical tests, e.g., anaerobic dissimilation of glucose, hydrolysis of gelatin, reduction of nitrate, and profuse growth on chloral hydrate-agar for the identification of presumptive isolates of *B. cereus*.

In a survey of selected dry food products in retail distribution, Kim and Goepfert (10) employed the MYP agar of Mossel et al. and reported that mannitol utilization by colonies appearing on this medium was not significantly helpful in eliminating non-*B. cereus* organisms and, indeed, made interpretation somewhat difficult when plates were held for 40 hr at 32 C. Moreover, the usefulness of acetylmethylcarbinol (AMC) production, NO_3 reduction, and starch hydrolysis was found to be somewhat questionable.

Preliminary experiments in this laboratory suggested that a serological test employing spore antigens might serve as a rapid confirmatory test for presumptive *B. cereus* isolates and prove valuable in epidemiological investigations of food

poisoning outbreaks. Such an approach necessitated the development of a selective (and differential) medium that would support the formation of free spores within the shortest possible period of time.

MATERIALS AND METHODS

Cultures. The strains of *B. cereus* employed in this study and their sources are listed in Table 1. Cultures were maintained on nutrient agar slants in screw-capped tubes at room temperature.

Production of turbidity in egg yolk and free-spore production. The degree of egg yolk turbidity and free-spore formation by *B. cereus* on the various media was assessed as follows. A loop was used to streak from agar slant cultures onto the surface of the test medium. Since these slant cultures varied in age from 1 week to several months, the inoculum consisted overwhelmingly of spores rather than vegetative cells. The test medium plates were incubated at 35 to 37 C for 16 to 24 hr. Organisms capable of eliciting turbidity on the medium evidenced this by formation of an opaque zone in the otherwise translucent pink-red medium. After incubation, the size of the zone of precipitate surrounding the isolated colonies was measured and recorded on a scale of 1 to 4+ (*see* footnote *a*, Table 3). A small portion of the center of the colony was picked, and this growth was emulsified in a drop of distilled water on a slide. The free-spore population was estimated to the nearest 10% after examination of the smear under dark-field illumination at ×1,250.

Composition of KG medium. The final composition of the egg yolk-polymyxin (KG) medium as it evolved during this study was as follows. The basal medium was prepared separately and consisted of 0.1% peptone, 0.05% yeast extract, 0.0025% phenol red, and 1.8% agar. The *p*H of the medium was 6.8. The basal medium was autoclaved at 121 C for 20 min. After sterilization and cooling to 50 C, 100 ml of sterile, concentrated egg yolk emulsion (Oxoid) and sufficient polymyxin B sulfate (Pfizer Co. Inc., Brooklyn, N.Y.) to result in a final concentration of 10 μg/ml were added to 900 ml of basal medium. The medium was poured into petri dishes, allowed to solidify, and stored in a manner to eliminate excess surface moisture. The complete medium can be stored for 1 week at 4 C before use. Longer storage is not recommended.

Food samples. The food samples employed in

TABLE 1. *Sources of Bacillus cereus cultures*

Strain	Supplier	Comment
T	R. S. Hanson, University of Wisconsin, Madison	
17 Benz, 27 Benz, 39 Benz, D_4	U. de Barjac, Institut Pasteur, Paris, France	Received as lecithinase-negative strains
B-47	A. Krieg, Institut für Biologische Schadlingsbekampfung, Darmstadt, Germany	Received as lecithinase-negative
2280	G. W. Gould, Unilever Research, Bedford, England	Received as lecithinase-negative
318, 319, 629, 1977, 1978, 1979, 1980	G. W. Gould, Unilever Research, Bedford, England	
331	G. W. Gould, Unilever Research, Bedford, England	Asporogenic mutant
5056	T. Midura, State Dept. of Public Health, Berkeley, Calif.	Isolated from food poisoning outbreak
F-22, F-42, F-66, F-71, F-77	C. V. Hall, Public Health Laboratories, Seattle, Wash.	Isolated from food poisoning outbreaks
B2-AC, B4-AC, B5-AC, B6-AC, B7-AC	D. A. A. Mossel, Louvain, Belgium	Isolated from food poisoning outbreaks
201	W. B. Sarles, University of Wisconsin, Madison	
C-14	Food Research Institute	

testing the sensitivity of the KG medium were purchased in retail outlets in Madison, Wis.

RESULTS AND DISCUSSION

During an earlier study (10), it was noted that many colonies that appeared on MYP agar plates evidenced a restricted (1+) zone of precipitate beneath the colony. The result was that many colonies were picked and subjected to extensive biochemical testing to ascertain that they were not weakly lecithinase-positive *B. cereus*. Additionally, two lecithinase-positive strains of the *polymyxa-macerans* group of *Bacillus* were also isolated. Various other species of *Bacillus*, e.g., *B. laterosporus*, *B. thuringinesis*, *B. mycoides*, and *B. anthracis*, have been reported to possess phospholipase C activity (16). Therefore, it was desirable to have a rapid confirmatory test for *B. cereus* organisms isolated from selective agar plates that employed the production of turbidity in egg yolk-agar as a differential system. Serological tests for species-specific spore antigens appeared to be suitable for this purpose (Kim and Goepfert, *unpublished data*). These tests necessitated the presence of free spores.

The first step was to determine whether a suitable population of free spores was present in colonies growing on MYP agar. Three colonies from each of 10 different MYP agar plates (representing different food samples) were examined after 24, 48, and 72 hr at 35 C. No free spores and very few endospores developed in the colonies within that period. Various substances [G-salts (3), 0.05% $MnCl_2$, and 0.05% sodium citrate] were added alone and in combination to MYP medium in an effort to enhance sporulation. None of the addends had an adverse effect on the lecithinase reaction of the test strain *B. cereus* T, but free spores could not be detected after 24 hr at 35 C.

Since the MYP medium contains a substantial amount of peptone (1%), the possibility existed that the medium was too nutritionally endowed, i.e., favorable to vegetative cell growth and not conducive to sporulation. Consequently, a basal medium containing polymyxin B, egg yolk emulsion, agar (1.5%), and phenol red was formulated. Phenol red was incorporated solely to facilitate observation of the zone of turbidity in the egg yolk medium. The basal medium alone and with various additions was tested for the ability to support ample lecithinase production and free-spore formation by the test strain *B. cereus* T (Table 2). Initially it appeared that lecithinase production and sporulation were mutually exclusive or only weakly compatible. Substances which stimulated lecithinase production (1% peptone) seemed to prohibit sporulation. This effect seemed not to be attributable to glutamate inhibition of sporulation, a phenomenon well documented by other workers (6), since the addition of 0.2% sodium glutamate did not affect the sporulation rate. Substances alone and in combination ($MnCl_2$, sodium citrate, ethylenediaminetetraacetic acid, and glucose) that supported sporulation seemed to preclude lecithinase production (or activity). Phosphorochloline (0.1%) was added to the plating medium to determine whether the end product of lecithinase activity would inhibit sporulation. This did not appreciably influence free-spore formation.

Finally, the incorporation of low levels of peptone and yeast extract into the basal medium resulted in lecithinase production and the formation of free spores by *B. cereus* T. Increasing the agar concentration from 1.5 to 1.8% enhanced free-spore formation by *B. cereus* T within the 24-hr incubation period at 37 C.

Twenty-five additional strains of *B. cereus* and

TABLE 2. *Effect of various substances on egg yolk turbidity and free-spore production by Bacillus cereus T*

Additions to basal medium[a]	Egg yolk turbidity	Free spore production (%)
None	2+	<10
Yeast extract + G salts	0	20
Yeast extract + 0.05% $MnCl_2$	0	20
Yeast extract + 0.05% sodium citrate	0	80
Yeast extract + 0.05% sodium citrate + 0.05% $MnCl_2$	0	80
Yeast extract + 0.05% sodium citrate + 0.05% $MnCl_2$ + 1.0% peptone	2+	0
Yeast extract + 0.05% sodium citrate + 0.2% sodium glutamate	0	40
Yeast extract + 0.05% $MnCl_2$ + 0.05% sodium citrate + 0.05% peptone	0	80
Yeast extract + 0.05% $MnCl_2$ + 0.01% EDTA[b]	0	80
Yeast extract + 0.05% $MnCl_2$ + 0.05% sodium citrate + 0.05 peptone + 0.1% glucose	0	50
0.1% Peptone	3+	50
Yeast extract	3+	60
Yeast extract + 0.1% peptone	4+	60
Yeast extract + 0.1% peptone + 0.3% agar	4+	>90

[a] Basal medium contained 10% (v/v) egg yolk emulsion, 0.0025% phenol red, 10 μg of polymyxin/ml, and 1.5% agar.

[b] EDTA, ethylenediaminetetraacetic acid.

TABLE 3. *Egg yolk turbidity and free-spore formation by certain Bacillus species on KG medium*

Organism	Egg yolk turbidity[a] at 24 hr	Free-spore population at 24 hr (% of total)
Bacillus cereus		
F-77	4+	80
5056	4+	10
B2-AC	4+	50
111 B4-AC	4+	<10
112 B5-AC	4+	80
113 B6-AC	4+	60
114 B7-AC	4+	80
201	4+	80
629	4+	20
1977	4+	90
1978	4+	90
1979	4+	90
1980	4+	90
C-14	4+	0
331 (asporogenic mutant)	4+	0
F-22	3+	10
F-71	2+	<10
D_4	2+	30
B-47	1+	10
17 Benz	1+	80
39 Benz	1+	10
27 Benz	1+	0
318	1+	90
319	1+	<10
2280	1+	<10
polymyxa-macerans		
G 13L	0	30
D 10L	0	30

[a] Egg yolk turbidity was graded as: 1+, precipitate restricted to area beneath colony; 2+, precipitate extending 0.5 to 1.0 mm around colony; 3+, precipitate extending 1 to 2 mm around colony; 4+, precipitate extending > 2 mm around colony.

the two lecithinase-positive *polymyxa-macerans* strains were streaked from pure cultures onto the complete medium. After incubation for 24 hr at 37 C, the plates were examined for evidence of turbidity around the colonies, and a small portion of a single colony of each strain was removed to a slide for examination by microscope. Preliminary experiments had disclosed that spore formation was greatest in that area of the plate where growth was heaviest. In the series of experiments reported here, the estimation of free-spore population was performed on colonies that were well isolated from the area of heavy growth. It is recognized that examination of a single colony does not yield data on the spore-forming performance of other colonies on the same plate, but the examination of a colony that was well isolated would appear to represent the minimum sporulating capacity under these given conditions. For this reason, examination of a single colony was accepted as representative. The data in Table 3 indicate that the KG medium was sufficient to encourage turbidity production and free-spore formation by most of the strains within the 24-hr period of incubation. It was possible to detect turbidity formation by the colonies quite readily after 16 hr at 35 to 37 C.

It was noted previously (Table 2) that $MnCl_2$-sodium citrate mixtures had a stimulatory effect on free-spore formation by *B. cereus* T. These substances were tested for their ability to enhance free-spore formation by some of the strains that did not form free spores readily on KG agar incubated at 35 to 37 C for 24 hr. This was accomplished by placing one drop of a solution containing 0.1% $MnCl_2$ and 0.1% sodium citrate (MC) on a 16-hr colony growing on KG agar. In addition to this, isolated colonies on the same plate that did not receive the solution were examined after 48 hr on KG medium (Table 4). The administration of MC solution to the colonies enhanced the free-spore formation by each strain except C-14 and 27 Benz. Incubation of untreated colonies for 48 hr resulted in increased free-spore production by all strains except 27 Benz. This strain exhibited a low level (~10%) of free-spore production only in colonies treated with MC and incubated for 48 hr. Untreated colonies at both 24 and 48 hr were observed to contain less than 10% endospores. The reasons for the failure to enhance free-spore formation

TABLE 4. *Enhancement of free-spore formation of selected strains of Bacillus cereus by extended incubation on KG agar and treatment with $MnCl_2$-sodium citrate (MC) solution*

Strain	Free spores formed (%)		
	No treatment[a]	$MnCl_2$-sodium citrate treatment[b]	No treatment[c]
F-71	<10	70	>90
B4-AC	<10	60	>90
39 Benz	10	30	>90
2280	<10	20	30
319	<10	50	>90
C-14	0	0	90
27 Benz	0	0	0

[a] Examined at 24 hr.

[b] $MnCl_2$-sodium citrate added at 16 hr. Examined at 24 hr.

[c] Examined at 48 hr.

TABLE 5. *Recovery of and free-spore production by Bacillus cereus T inoculated into selected sterile food slurries*

Food slurry[a]	Count/ml on KG agar	Free-spore formation (%)
Potato product..	97×10^3	60
Gravy mix.....	147×10^3	50
Seasoning mix...	125×10^3	20

[a] Each slurry received 131×10^3 *B. cereus* T spores per ml as determined by plate count on nutrient agar of the spore suspension used as the source of inoculum.

by 27 Benz are not known. The success of the MC treatment in enhancing free-spore production within 8 hr after application indicates that in the great majority of cases serological analyses can be initiated (and completed) on the second working day. It is suggested that MC not be applied routinely to colonies appearing on KG agar but that it be administered only if initial examination by microscope reveals a lack of spore formation. Efforts to incorporate MC into the KG agar and thus eliminate the cumbersome process of addition directly to the colonies have failed because of the inhibition of the lecithinase reaction by this solution. It should also be noted that the lecithinase reaction of the two *polymyxa-macerans* strains was inhibited on the KG agar under the test conditions employed. This would indicate an increased specificity for *B. cereus* organisms.

To determine whether food materials would affect the isolation and recognition of *B. cereus* on KG medium, a series of trials employing food materials was undertaken.

Initially, a suspension of spores of *B. cereus* T was prepared, diluted in peptone, and inoculated into autoclaved 1:10 aqueous suspensions of several food materials. A dried potato product, gravy mix, and seasoning mix were employed. The recovery and free-spore production data are presented in Table 5. At this level of inoculum (1.3×10^3/ml), it appeared that the sterile food slurry had little or no effect on the recovery of *B. cereus* T. However, free-spore production may have been influenced by the food constitutents of the slurry even though these were diluted significantly by the plating procedure.

Five additional strains of *B. cereus* (all isolated from food poisoning outbreaks) were then inoculated into nonsterile 10% aqueous slurries of four food prototypes. The recovery rate of these strains on KG agar was calculated by comparing the KG agar counts with nutrient agar counts made on the cell suspension serving as the inoculum for the slurries (Table 6). The recovery of the five strains on the KG medium ranged from 63.8 to 276.7% of the organisms inoculated into the food prototypes.

This efficiency of recovery of relatively high numbers of *B. cereus* prompted a pilot study to determine the effectiveness of the KG medium in recovering low numbers of *B. cereus* from naturally contaminated retail food products. Six food prototypes were chosen, and 10-g samples were aseptically transferred to 90 ml of 0.1% peptone-water. Samples (0.1 ml) of the 10^{-1}, 10^{-2}, and 10^{-3} dilutions were surface-plated in duplicate on KG agar and MYP agar. Representative colonies of lecithinase-producing organisms that appeared on KG agar were picked after incubation at 35 to 37 C for 22 hr for examination by microscope and estimation of free-spore populations (Table 7). *B. cereus* colonies were observed on both MYP and KG agar plates from 21 of 26 food samples. Three samples yielded colonies on MYP agar but not on KG agar. Two samples were positive on KG agar but not on MYP agar. Fourteen samples yielded higher *B. cereus* counts on KG agar, eight samples were higher on MYP agar, and in four samples identical counts were obtained on both media. Based on these data, it seems safe to assume that the two media were comparable in terms of ability to recover low numbers of *B. cereus*.

TABLE 6. *Recovery on KG agar of food poisoning strains of Bacillus cereus inoculated into nonsterile food slurries*[a]

Food product	*B. cereus* strain no.				
	B2-AC	B5-AC	5056	F-42	F-66
Dried potatoes					
KG agar count/ml[b]	5.57	3.27	2.27	6.77	2.47
Per cent recovery	74.2	234.7	169.3	123.0	166.8
Gravy mix					
KG agar count/ml[b]	4.79	2.09	3.69	6.59	2.89
Per cent recovery	63.8	149.2	276.1	119.8	195.2
Spaghetti sauce					
KG agar count/ml[b]	5.99	2.99	2.01	4.19	1.69
Per cent recovery	79.8	213.5	150.0	76.1	107.4
White pepper					
KG agar count/ml[b]	5.29	1.39	3.59	6.59	2.79
Per cent recovery	70.5	99.2	276.7	119.8	188.5

[a] Number of cells inoculated per milliliter of slurry: strain B2-AC, 7.5×10^5; strain B5-AC, 1.40×10^5; strain 5056, 1.34×10^5; strain F-42, 5.50×10^5; and strain F-66, 1.48×10^5.

[b] Values to be multiplied by 10^5.

TABLE 7. *Comparison of recovery of Bacillus cereus from naturally contaminated food samples by surface plating on KG and MYP agar*

Food product	Sample	MYP agar count/gram[a]	KG agar count/gram	Free-spore production on KG agar in colonies (%)
Dried potatoes	A2	100	100	20
	A4	2,000	200	70
	A3	400	500	10
	A13	800	900	70
	A16	4,000	1,800	20
	A17	400	250	70
Gravy mix	C14	100	100	0
	C19	<100	100	NT[b]
Spaghetti sauce mix	D1	100	100	50
	D10	100	100	0
	D12	100	300	20
	D13	200	300	NT
Spices	F16	100	200	20
	F18	200	<100	
	F22	200	800	NT
	F11	<100	100	10
	F23	400	<100	
	F24	900	1,700	NT
Seasoning mixes	G1	500	900	10
	G3	1,000	300	80
	G5	400	200	0
	G7	200	800	90
	G17	1,000	1,200	30
	G18	400	2,300	90
	G20	100	<100	
Flour	I1	100	400	0

[a] Incubated at 32 C.
[b] Not tested.

Examination of colonies appearing on KG agar from 19 of the 26 samples showed that 15 of the 19 had produced a sufficient number of free spores to be detectable by microscope. Unfortunately, the plates containing the free-spore "negative" colonies were not kept an additional 24 hr and reexamined. Moreover, these colonies were not treated with MC solution to enhance sporulation. Thus, it is not known whether these treatments would have affected detectable free-spore formation. In addition, it is not known whether (i) these organisms were asporogenic or oligosporogenic mutants (unlikely), (ii) sporulation was influenced by food constitutents on the plate (likely), or (iii) these were normally slow sporulating cultures.

The results indicate that KG agar is as efficient as MYP agar in recovering and presumptively identifying *B. cereus* organisms from food products. Lecithinase reactions by known *B. cereus* cultures and food contaminants are comparable on each medium. Nygren (13) has postulated that lecithinase-negative mutants of *B. cereus* are not pathogenic. However, in light of recent studies on the role of lecithinase in food poisoning by *Clostridium perfringens* (9), it appears too early to judge the pathogenicity of lecithinase-negative *B. cereus* strains. For this reason, we concur with the suggestion of Mossel et al. (12) that lecithinase-negative egg yolk turbidity-positive colonies of appropriate morphology be picked for confirmatory testing. Based on our earlier experience with biochemical tests [e.g., anaerobic glucose dissimilation, nitrate reduction, acetylmethylcarbinol production, and gelatin and starch hydrolysis (10)], we cannot agree that these time-consuming and often erratic tests are the confirmatory methods of choice. Preliminary data in this laboratory have indicated that serological reactions involving certain spore antigens are more rapid, sensitive, and specific than biochemical tests. Therefore, it would be extremely beneficial to have a plating medium that would not only indicate presumptive *B. cereus* colonies but also induce these organisms to sporulate within the 24-hr incubation period. The KG medium as presently formulated, although not 100% effective in inducing free spores detectable by microscope, has achieved the desired result in the majority of trials.

It is hoped that the existence of two comparable, easily prepared and interpreted selective media for the isolation of *B. cereus* will prompt those laboratories currently not examining food samples or suspect food poisoning vehicles for these organisms to do so. Only in this manner will the necessary data be generated to ascertain whether this organism is the cause of a significant number of foodborne outbreaks in this country. Current knowledge about *C. perfringens* food poisoning, compared to its known role in foodborne disease 15 years ago, is an outstanding example of what a systematic, scientific approach can add to our knowledge of public health hazards.

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