Enumeration of *Bacillus cereus* in Foods

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For the enumeration of vegetative cells and spores of *Bacillus cereus* in foods, a mannitol-egg yolk-phenol red-agar has been developed which exploits the failure of *B. cereus* to dissimilate mannitol, and the ability of most strains to produce phospholipase C. When a high degree of selectivity was required, polymyxin B sulfate in a concentration of 10 ppm appeared to be the most effective selective additive. Useful characteristics for the identification of presumptive isolates of *B. cereus* were found to be: morphology, dissimilation of glucose mostly to acetyl methyl carbinol under anaerobic conditions, hydrolysis of starch and gelatin, reduction of nitrate, and growth on 0.25% chloral hydrate agar.

Bacillus cereus has been incriminated in outbreaks of food poisoning in The Netherlands as it has elsewhere. Mashed potatoes and vegetables, minced meat, liver sausage, Indonesian rice dishes, puddings, and soups (5) appear to be the causative foods in most cases in our country. Although the definite proof is lacking that, as postulated by Nygren (21), phosphoryl choline formed during prolific growth of *B. cereus* in certain foods is the ultimate cause of the clinical phenomena, it has been established that very high numbers of cells, i.e., a minimum of $10^7/g$ of food (1, 5, 7, 18), are required to cause outbreaks of disease—except in young children, where as few as $10^5/g$ may suffice (3). Hence, it is of interest, to be able to follow the proliferation of *B. cereus* in foods, so as to establish, before any disease may occur, that the food may become dangerous upon further storage.

No specific medium for the enumeration of *B. cereus* in foods has been previously described. This is probably due to the fact that, where *B. cereus* food poisoning has occurred, the numbers of viable cells per gram of food are so high that isolation of the organism presents no problems. Also, the examination of food ingredients for *B. cereus* has usually been carried out by subjecting dilutions to some form of preliminary heat treatment and then plating out, which, again, is invariably a successful procedure (14).

Heating suspensions of foods or food ingredients with the purpose of eliminating most other bacteria prior to determining *B. cereus* is not a generally valid procedure, since it might destroy most of the organisms, e.g., when only very few cells have formed spores. It has been demonstrated by Nikodemusz (18), and since fully confirmed by ourselves, that the sporulation of populations of *B. cereus* in foods may be very low;

occasionally, as few as one spore-bearing cell or spore was encountered in approximately 10^4 total viable cells of *B. cereus*. Therefore, an attempt was made to develop a selective and differential medium for the enumeration of spores plus vegetative cells of *B. cereus* in the presence of much higher levels of other bacteria in foods.

MATERIALS AND METHODS

Strains. Sixteen authentic strains of *B. cereus* were used in this study. Among these were the Smith (22) strain (at present ATCC 14579), obtained in 1955 from the late A. J. Kluyver, Delft, The Netherlands; the original food-poisoning strain (7), obtained from S. Hauge, Oslo, Norway; two strains causing "bitty cream" in milk and three isolated from processed cheese, received from T. Galesloot, Ede, The Netherlands; and nine strains that had been involved in food-poisoning outbreaks in The Netherlands, made available by M. van Schothorst, Utrecht, The Netherlands.

Before use, these strains were purified by streaking onto plates of blood-agar (7) and then were transferred onto Brain Heart Infusion Agar slants.

Differential medium. A critical review of the properties of the aerobic sporeforming bacteria reported in the literature (11, 13, 23) resulted in an attempt to exploit two compatible properties of *B. cereus*, i.e., the production of phospholipase C and failure to dissimilate arabinose, xylose, or mannitol. Because of its very much greater thermostability, the latter polyol was chosen rather than the pentose sugars.

An agar medium (MY-agar) of the following composition was ultimately chosen: meat extract, 1 g; peptone, 10 g; D-mannitol, 10 g; NaCl, 10 g; phenol red, 25 mg; agar, 15 g; water, 900 ml; *p*H, 7.1. After this basal agar was sterilized and cooled to 49 ± 1 C, to 90 ml of agar 10 ml of a 20% egg yolk emulsion prepared according to Billing and Luckhurst (2), e.g., as marketed by Oxoid, Ltd., London, England, was added.

Selective media. Three selective agents were investi-

gated. These included sodium chloride, because we had quite often, in counting *Staphylococcus aureus* in foods by the use of Chapman's 7.5% salt-agar (4), encountered numerous colonies of bacilli, among which *B. cereus* occasionally occurred; NaCl was tried in concentrations of 7.5 and 5.0% (w/w).

The next selective agent studied was ethyl alcohol, because Nikodemusz (19) had found that 8% of this alcohol inhibited some of the most seriously interfering food bacteria. Ethyl alcohol was always added to the agar base, cooled to 48 C. Plates were then rapidly inoculated and incubated in closed, but not evacuated, anaerobic jars to prevent excessive evaporation of the selective agent.

Finally, polymyxin B sulfate was tried, as suggested by Donovan (6), since it could indeed be expected (8, 16) to inhibit effectively many bacteria other than the *Bacillaceae* in foods. A solution containing 50 mg of polymyxin B sulfate (Chas. Pfizer & Co., Inc., New York, N.Y.) per 50 ml of water was prepared, sterilized by filtration, and added in 1-, 2-, 5-, and 10-ml quantities to 100 ml of basal agar, yielding final concentrations of 10 to 100 μg/ml.

Counting technique. When examining pure cultures, suspensions were prepared that contained in the order of 2×10^2 to 5×10^2 viable cells of *B. cereus* per ml. Quantities of 0.1 ml of these suspensions were deposited in the center of 15-cm diameter plates filled with the agar under investigation and then evenly spread over the surface, by use of a sterile, glass, hockey stick-shaped spatula (4 by 12 cm). Incubation was for 18 to 40 hr at 32 C. Suspensions were examined in the same way with Brain Heart Infusion Agar as the medium.

Foods were first of all diluted 10^{-1} to 10^{-4} with peptone-saline (24). Quantities of 0.1 ml of these dilutions were then spread over dried MY-agar plates and incubated for up to 40 hr at 32 C. Rough, dry colonies with a distinct, violet-red background, surrounded by a halo of dense white precipitate, were taken as the presumptive viable count of vegetative cells plus spores of *B. cereus*.

Confirmation procedures. A number of colonies equal to the square root of the total number of colonies obtained, taken at random, was first purified on MYP-agar. Isolated colonies so obtained were next examined for cell shape and spore topography in Gram-stained preparations.

Subsequent biochemical confirmation reactions comprised the following: aerobic and anaerobic dissimilation of glucose, D-mannitol, and xylose, by use of Mossel and Martin's single-tube modification of Hugh and Leifson's O/F-technique (17); reduction of nitrate in nutrient broth containing 0.1% of analytical reagent-grade KNO_3 incubated up to 6 days; dissimilation of gelatin by Frazier's technique (17); amylase production, judged by the formation of a halo round colonies on Nutrient Agar plus 1% soluble starch, developed by flooding with Lugol solution; production of acetyl methyl carbinol in MR-VP broth (Difco) and in the phosphate-free glucose broth of Smith et al. (23) incubated for up to 20 days; growth on Knisely (12) 0.25% chloral hydrate agar; and growth in 10% NaCl broth according to Kundrat (13). All these tests were carried out at 32 C.

TABLE 1. *Enumeration of 10 type strains of Bacillus cereus on infusion agar (TDYM) vs. MY-agar plus 5% NaCl*

Strain no.	Count per ml of suspension on	
	TDYM-agar	MY-agar plus salt
1	0.5×10^8	0.1×10^7
2	0.1×10^9	$< 10^7$
3	0.2×10^9	0.4×10^8
4	0.3×10^9	0.3×10^8
5	0.3×10^8	0.4×10^8
6	0.4×10^7	0.5×10^6
7	0.9×10^7	0.6×10^7
8	1.0×10^8	0.6×10^8
9	1.0×10^8	0.6×10^8
29	0.3×10^9	0.2×10^9

RESULTS AND DISCUSSION

Comparison of various inhibitors used in MY-agar. Comparative counts of the 16 type strains on 7.5% NaCl-agar, 8% ethyl alcohol-agar, and 20 to 100 μg/ml polymyxin agar, and on infusion agar were so inconsistent that it soon became evident that these selective agents were of little use. MY-agar containing only 5% NaCl was much better as is shown by the data in Table 1. Yet, counts on this agar of the order of $\leq 10\%$ of the count on infusion agar occurred with about half of the strains tested.

MY-agar containing 10 μg/ml of polymyxin (MYP-agar) performed much better (Table 2). This medium was, therefore, studied in more detail, in the examination of commercial food and meal samples.

Use of MY-polymyxin agar in routine food microbiology. This agar has now been used for almost 2 years in daily practice. The foods most frequently examined by this technique were pudding powders, cocoa, dried meals, dried porridges, cooked sausages, meat balls and croquettes, various Indonesian rice dishes, meat and vegetable salads, frozen desserts, and animal feeds. A total of nearly 250 samples have been examined.

The plates worked surprisingly well under practical conditions. Even when the ratio of *B. cereus* to total population including staphylococci was as low as $1:10^6$, the *B. cereus* could be easily recognized and isolated for further study. The presumptive counts of *B. cereus* in all samples were, in fact, relatively low; i.e., they usually did not exceed 10^3/g. These results are in full agreement with surveys carried out in Scandinavia (21), Hungary (20), Germany (L. Nieper, Dissertation, Tierärztl. Hochschule, Hannover, Germany, 1964), and Rumania (10).

Study of typical colonies cultured from foods.

TABLE 2. *Enumeration of 10 type strains of Bacillus cereus on infusion agar (TDYM) vs. MY-agar plus 10 ppm of polymyxin*

Strain no.	Count per ml of suspension on	
	TDYM-agar	MY-agar plus polymyxin
1	0.3×10^9	0.2×10^9
2	0.9×10^8	0.7×10^8
3	0.2×10^9	0.1×10^9
4	0.2×10^9	0.1×10^9
5	1.0×10^8	0.9×10^8
6	0.4×10^8	0.5×10^8
7	1.0×10^8	0.8×10^8
8	0.8×10^8	0.7×10^8
9	0.3×10^9	0.3×10^9
29	0.6×10^8	0.4×10^8

Typical colonies obtained from such foods, when studied as described in the previous section, could virtually always be confirmed as *B. cereus*. The question presented itself, though, as to the extent to which an organism had to show the properties listed by Smith, Gordon, and Clark (23) before it could be considered to be *B. cereus*. In an attempt to answer this question, the 16 type strains were examined for the most consistent criteria listed. It appeared that all strains fermented glucose under anaerobic conditions, did not attack D-mannitol or xylose, dissimilated gelatin, and reduced nitrate. Five strains failed to attack soluble starch, and five did not produce acetyl methyl carbinol, even when the method recommended by Smith et al. (23) was used. All strains grew profusely on Knisely's chloral hydrate agar, but only eight developed well in Kundrat's 10% NaCl broth.

A striking result was that two strains, isolated by Galesloot from processed cheese, did not possess a phospholipase C. These strains were also amylase-negative. Ionescu et al. (9) did not encounter lecithinase-negative isolates of *B. cereus*, but Kundrat did; it may well be that the less refined technique of McGaughey and Chu (15) which Kundrat applied was insufficiently sensitive. One could, of course, reason that lecithinase-negative mutants of *B. cereus* may not be of very much interest with respect to food poisoning in view of Nygren's theory of the pathogenesis of this disease. However, this theory needs more experimental confirmation before one is entirely justified in discarding lecithinase-negative strains.

Pending the definite substantiation of Nygren's postulate, it might, therefore, be wise to study also a reasonable proportion of egg yolk-negative, mannitol-negative *Bacillus* colonies cultured from

foods on MYP-agar. When these are found morphologically typical, they should be submitted to the tests which were found invariably positive for *B. cereus*: anaerobic dissimilation of glucose, gelatin liquefaction, nitrate reduction, and profuse growth on chloral hydrate agar. Obviously these extra steps need not be undertaken when foods or meals already show clinically significant, alarming, or potentially dangerous numbers of lecithinase-positive isolates.

LITERATURE CITED

1. ADAM, W. 1958. Die quantitative bakteriologische Lebensmitteluntersuchung bei der Diagnose der unspezifischen Lebensmittelvergiftungen sowie der Lebensmittelüberwachung. Arch. Hyg. Bakteriol. **142**:232–240.
2. BILLING, E., AND E. R. LUCKHURST. 1957. A simplified method for the preparation of egg yolk media. J. Appl. Bacteriol. **20**:90.
3. BODNAR, S. 1962. Ueber durch Bac. cereus verursachte alimentäre atypisch verlaufende Lebensmittelvergiftungen. Z. Ges. Hyg. Grenzgeb. **8**: 388–390.
4. CHAPMAN, G. H. 1945. The significance of sodium chloride in studies of staphylococci. J. Bacteriol. **50**:201–203.
5. CLARENBURG, A., AND E. H. KAMPELMACHER. 1957. Bacillus cereus als oorzaak van voedselvergiftiging. Voeding **18**:384–386.
6. DONOVAN, K. O. 1958. A selective medium for Bacillus cereus in milk. J. Appl. Bacteriol. **21**: 100–103.
7. HAUGE, S. 1955. Food poisoning caused by aerobic spore-forming bacilli. J. Appl. Bacteriol. **18**:591–595.
8. HIRSCH, H. A., C. G. McCARTHY, AND M. FINLAND. 1960. Polymyxin B and colistin: activity, resistance and cross-resistance in vitro. Proc. Soc. Exptl. Biol. Med. **103**:338–342.
9. IONESCU, G., C. IENISTEA, AND C. IONESCU. 1965. Frecventa B. cereus in carne tocata si mezeluri. Microbiol. Parazitol. Epidemiol. (Bucharest) **10**:327–334.
10. JANTEA, F., P. MILOSESCU, E. BISTRICEANU, AND D. BAD-OPRISESCU. 1965. Incidenta Bac. cereus in preparate culinare. Microbiol. Parazitol. Epidemiol. (Bucharest) **10**:163–168.
11. KNIGHT, B. C. J. G., AND H. PROOM. 1950. A comparative survey of the nutrition and physiology of mesophilic species in the genus Bacillus. J. Gen. Microbiol. **4**:508–538.
12. KNISELY, R. F. 1965. Differential media for the identification of Bacillus anthracis. J. Bacteriol. **90**:1778–1783.
13. KUNDRAT, W. 1963. Zur Differenzierung aerober Sporenbildner (Genus Bacillus Cohn). Zentr. Veterinaermed **10**:418–426.

14. LABOTS, H., AND G. HUP. 1964. Bacillus cereus in rauwe en gepasteuriseerde melk. I. Bepaling van het aantal B. cereus-sporen in melk. Nederl. Melk Zuivel Tijdschr. **18**:112–126.

15. MCGAUGHEY, C. A., AND H. P. CHU. 1948. The egg-yolk reaction of aerobic sporing bacilli. J. Gen. Microbiol. **2**:334–340.

16. MOSSEL, D. A. A. 1959. Enumeration of sulphite-reducing clostridia occurring in foods. J. Sci. Food Agr. **10**:662–669.

17. MOSSEL, D. A. A. 1962. Attempt in classification of catalase-positive staphylococci and micrococci. J. Bacteriol. **84**:1140–1147.

18. NIKODEMUSZ, I. 1958. Bacillus cereus als Ursache von Lebensmittelvergiftigung. Z. Hyg. Infektionskrankh. **145**:335–338.

19. NIKODEMUSZ, I. 1962. Ueber einen neuen Elektivnährboden, der zur Isolierung von Bacillus cereus aus Mischflora geeignet ist. Arch. Lebensmittelhyg. **13**:56–58.

20. NIKODEMUSZ, I., M. BOJAN, V. HOCH, M. KISS, AND P. KISS. 1963. Das vorkommen von Bac. cereus in Lebensmitteln. Arch. Lebensmittelhyg. **14**:172–173.

21. NYGREN, B. 1962. Phospholipase C-producing bacteria and food poisoning. An experimental study on Clostridium perfringens and Bacillus cereus. Acta Pathol. Microbiol. Scand. **56**: Suppl. 160.

22. SMITH, N. R., T. GIBSON, R. E. GORDON, AND P. H. A. SNEATH. 1964. Type cultures and proposed neotype cultures of some species in the genus Bacillus. J. Gen. Microbiol. **34**:269–272.

23. SMITH, N. R., R. E. GORDON, AND F. E. CLARK. 1952. Aerobic sporeforming bacteria. U.S. Dept. Agr. Monograph No. 16.

24. STRAKA, R. P., AND J. L. STOKES. 1957. Rapid destruction of bacteria in commonly used diluents and its elimination. Appl. Microbiol. **5**:21–25.