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A procedure for estimating <u>Bacillus cereus</u>

in soil and stream-sediment samples

by

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ABSTRACT

Among the aerobic spore-forming bacilli, only Bacillus cereus and closely related strains produce an opaque zone in egg-yolk emulsion agar. This characteristic, also known as the Nagler or lecitho-vitellin reaction, has long been used to rapidly identify and estimate presumptive B. cereus. The test is here adapted to permit estimation of B. cereus spores in soil and stream-sediment samples. Relative standard deviation was 10.3% on counts obtained from two 40-replicate pour-plate determinations. As many as 40 samples per day can be processed. Enough procedural detail is included to permit use of the test in conventional geochemical laboratories. The presence of B. cereus spores appears to be related to several types of concealed mineral deposits, including vein and disseminated gold deposits.

INTRODUCTION

The purpose of this paper is to provide details of a procedure for estimating B. cereus group organisms in soil and stream-sediment samples. Empirical studies of the usefulness, in prospecting, of B. cereus spore distributions, as determined by this test, were begun in 1983 as a result of finding anomalous numbers of the spores of this organism in soils overlying an exposed porphyry copper deposit (Watterson and others, 1984).

The idea that microorganisms might be of use in exploring for mineral deposits has been around for many years. After the Soviet report (Mogilevskii, 1938) that methane-oxidizing bacteria can serve as indicators of petroleum deposits, Mogilevskii's student, G. P. Slavnina (1957), suggested that bacteria might also be of use in prospecting for metal deposits. An account of the the development of this neglected petroleum-prospecting technology was given by Davis (1967), and recently discussed by Updegraff (1985). L. E. Kramarenko (1962) and A. K. Lisitsyn and E. C. Kuznetsova (1967) reported studies of microbial biocenoses in underground waters associated with mineral deposits. Both reports illustrated the usefulness of microbiological studies in understanding the geometry and evolution of ore deposits. Kramarenko (1962) specifically recommended the use of microorganisms in mineral exploration. Lyalikova (1974, 1978) has studied the characteristics and distribution of an antimony-oxidizing organism in the vicinity of antimony deposits, but has not recommended it for prospecting. Miller (1983) has shown that the distribution of thiobacilli in soil corresponds to concealed sulfur occurrences in west Texas. Letunova and Koval'sky (1978) published a monograph, "The geochemical ecology of microorganisms," which summarized extensive research concerning the adaptation of a variety of microorganisms to their geochemical environment. Thus, accumulating data indicate soil microflora are highly adapted to their geochemical environment. At the suggestion of H. W. Lakin (1979), a project was set up within the U. S. Geological Survey to investigate the possiblity of geomicrobiological exploration. Most recently, papers by H. L. Ehrlich, R. R. Colwell, D. M. Updegraff, A. O. Summers, G. Stotzky, B. H. Olson, and the author consider various aspects of this possible application (Carlisle and others, 1985). The study of Clark and Watterson (1984) is apparently the first of a microorganism whose distribution appears to indicate non-petroleum mineral deposits.

Watterson and others (1984) have recently reported that the percentages of soil Bacillus species (spp.) able to resist low dosages of penicillin in test media correlate significantly (P<.01) with the distribution of a number of soil metals in the vicinity of two mineral deposits. Further study of the Poorman Creek deposit (Watterson and others, 1984) and subsequent studies of the Cotter Basin Prospect (Watterson and others, 1983a and b; Watterson and others, unpublished data) have revealed that the distribution of one organism, B. cereus, the most penicillin resistant of the Bacillus group (Curran and Evans, 1946), as estimated in egg-yolk agar, statistically explains (P<.001) the penicillin-resistance data at the Poorman Creek and Cotter Basin deposits. This finding suggested that the distribution of B. cereus in the vicinity of other known mineral deposits should be compared to other available geochemical and geological data.

On this basis, all available recently collected soil and stream-sediment samples (which had not been oven dried) have been assayed for B. cereus. B. cereus determinations on several hundred soil and stream-sediment samples, collected in a wide range of North American environments, indicate that at least a few viable B. cereus spores per gram can be recovered from most soil and stream-sediment samples. Stream-sediment samples appear to contain "background" counts ranging from zero to a few dozen viable B. cereus spores per gram. Soil samples in all but one of the preliminary study areas contain "background" counts ranging from none to a few hundred spores per gram. Counts as high as 10^6 and 10^4 viable B. cereus spores (colony-forming units, CFU's) per gram have been encountered in soils and stream-sediment samples, respectively. In several study areas, the population distribution of the organism appears to coincide with the distribution of ore minerals and/or ore-associated element dispersion patterns (Watterson and others, 1983b; Clark and Watterson, unpublished data; Watterson and others, unpublished data; Parduhn and Watterson, unpublished data). Several environments have been studied, including coastal wetlands in the Alaskan arctic, an alpine forest in Colorado, a grassland area near Globe Arizona, and desert environments in Utah and Nevada. The highest counts of B. cereus so far encountered (>10^6 per gram at 30-40 cm depth) were found in soils developed on more than 100 meters of overburden over a drill-proven segment of the Dee disseminated gold deposit, Elko County, Nevada (Watterson and others, in press). As suggested by the data of Mishustin and Mirsoeva (1968), it may be that large areas exist in which conditions unrelated to mineralization may be favorable for the development of large populations of this organism. Indeed, it seems extraordinary that a common soil microorganism should be an indicator microorganism. This is contrary to experience with indicator plants, which are invariably rare. Thus, for the time being, it is recommended that assays for this organism should be conducted on an experimental basis until sufficient data are accumulated to permit an adequate assessment of its practical usefulness in mineral exploration. The purpose of this report is to describe the procedure currently being used to study the distribution of presumptive B. cereus in soil and stream sediments. An attempt has been made to include enough detail to encourage the concurrent investigation of this organism in other geochemical laboratories. It should be emphasized that the test has been implemented and is being used for empirical rather than taxonomic purposes. The organisms so identified can be designated B. cereus on a probabilistic basis. Random egg-yolk-positive isolates, however, have all proven to be B. cereus according to tests recommended in the simplified key of Norris and others (1981).

Suggested sample collection procedure

Samples intended for B. cereus assay are most conveniently collected under dry conditions and sieved at the sample site to -30 mesh with an aluminum-frame, stainless steel sieve. Sterile procedure is neither necessary nor desirable. In forest settings, remove loose forest mull and, using a garden hoe, dig up and mix the first 10 cm of soil in an area comprising about 900 cm^2. After sieving, store the sample in a heavy-duty, paper sample bag which will permit water to evaporate. Between sample sites, it is only necessary to remove visible soil from the sieve and hoe. This is because the same dilution mathematics applies to samples collected for the study of (spore-forming) bacterial populations as to samples collected for geochemical analysis. In desert or other environments subject to eolian contamination, it may be desirable to remove at least several centimeters of soil prior to sampling. However, care should be taken to sample the same depth at each site, as the soil bacterial population is a sensitive function of depth in most soils (Waksman, 1936). Under damp or wet conditions, allow the samples to air dry, out of direct sunlight if possible, prior to sieving, in order to allow vegetative cells to sporulate naturally. Because stream-sediment samples generally contain fewer spores than soils, the 60- or 80-mesh sieves commonly used in preparing stream-sediment samples concentrate spores to some extent and are therefore preferable to a 30-mesh sieve. Counts appear to be little affected by grinding in a vertical ceramic-plate mill, but grinding is undesirable as it necessitates additional centrifuging. Prior to bringing the samples into the laboratory, it is convenient to transfer 20 to 50 grams of sample to a standard cardboard sample carton. Under cool, dry storage conditions, B. cereus counts can be expected to change little over a period of several years. Sneath (1962) summarized data showing that the death-rate of B. cereus spores in experimentally dried soils is no more than about one logarithm of their original count per gram per 50 years. For this reason, samples collected many years in the past can be examined for their relative content of B. cereus spores, again provided they were not oven dried. This circumstance should permit a relatively rapid assessment of the technique through comparison of B. cereus counts to data already assembled on existing soil and stream-sediment sample sets.

Precautions

The laboratory should be as clean and free from dust and turbulent air as possible. Laboratory surfaces and equipment can be routinely sterilized by spraying with 70% ethanol and wiping down with disposable tissues. As a routine control measure, sterile empty petri dishes should be periodically opened on laboratory benches and after various intervals poured with egg-yolk agar to monitor the occurrence of possible B. cereus and other organisms in laboratory air. All used petri dishes and other items containing cultivated microorganisms should be sterilized in autoclave bags prior to disposal. In the absence of an autoclave, adequate sterilization can be carried out by leaving autoclave bags in pans in a 100°C drying oven overnight. Investigators should be aware that B. cereus, like a number of other soil microorganisms, can cause food poisoning and has been associated with several types of infections (Norris and others, 1981). Laboratories routinely using this or other microbiological procedures should consult, post, and observe safety precautions published in standard microbiological laboratory manuals, e.g., the current ASM manual (Gerhardt and others, 1981).

Laboratory equipment

Four essential items of laboratory equipment include a pressure cooker or (preferably) autoclave, a circulating constant-temperature water bath, a laboratory balance capable of weighing to +0.01 g, and a mechanical shaker. An incubator, a refrigerator, and a small centrifuge are desirable but not absolutely necessary. Equipment and other disposable supply items with commercially available examples are listed below:

autoclave	Market Forge Sterilmatic Model STM-E Type C
laminar flow hood	Environmental Air Control, no. TT4830
circulating water bath	Precision Scientific, W 3085-5
incubator	Lab Line, J 1631
16x150 mm screw cap culture tubes	American Scientific Products, no. T 1354-14
1.1 ml pipettes	Kimble, 56400
5 ml pipettes	Falcon, 7529
10 ml pipettes	Falcon, 7530
250 ml media bottles	Wheaton-Kimbler, 219577
petri dishes	Van Waters & Rogers, 253840-070
autoclave bags	American Scientific Products, A 9500-12
glucose (Difco)	American Scientific Products, 0155-17
yeast extract (Difco)	American Scientific Products, 0127-02
agar (Difco)	American Scientific Products, 0140-01

B. cereus enumeration procedure

One-gram samples of soil or stream sediment are aseptically weighed into 16 x 150 mm, screw-cap culture tubes containing 9 ml sterile deionized water. This sterilization is accomplished by autoclaving tightly capped tubes at 121°C for 15 minutes. Aseptic weighing procedure: after autoclaving in aluminum foil, transfer weighing papers to sterile petri dishes and remove them as needed with alcohol-wiped tweezers. Periodically clean the weighing surface with 70% ethanol. This does not kill all bacterial spores, but removes dust and significantly reduces spore numbers. Use an alcohol-wiped weighing spoon to transfer the sample to a new weighing paper. Lift the corners of the weighing paper and slide the sample into the tube. Samples are then shaken on a mechanical shaker for exactly 10 minutes, heat-treated in 90°C water for one minute, immediately cooled in tap water, and, if necessary, centrifuged at 1200 rpm in an anglehead benchtop centrifuge for 3 minutes. Experimentation has shown that this procedure does not significantly reduce spore counts, and effectively removes most of the soil debris that interferes with plate counts. The 90°C, 1-minute heat treatment has been shown by lengthy experimentation to adequately pasturize natural samples and to optimize ratios of B. cereus to other bacilli recovered. Two additional serial 1/10 dilutions of soil samples (only one additional dilution is necessary in the case of most stream-sediment samples) are made in sterile 9 ml deionized water blanks using 1.1 ml sterile, disposable polystyrene pipettes. Experience has shown that significant variation can be introduced, if intervals between weighing, heat treatment, centrifuging, dilution, or pour plating exceed more than a few hours. It is desirable, but not necessary to carry out transfer operations in a sterile laminar-flow hood. One-ml aliquots of each dilution are transferred to the center of previously marked 100 x 15 mm sterile disposable petri dishes. After briefly agitating the petri dish to

resuspend settled material contained in the 1-ml drop, approximately 8 ml of 47°C egg-yolk agar (EYA) medium are poured directly on the aliquot drop, and the petri dish is gently tilted and shaken back and forth to form a thin, homogenous layer containing evenly distributed soil particles and spores. After the agar solidifies, the petri dishes are inverted and arranged in stacks of three on countertops or in a 30°C incubator for approximately 15 hours. Countertop incubation is equally satisfactory but requires about 21 hours. Egg-yolk positive colonies are generally visible after 15 hours and are revealed by a uniform, circular opaque zone 2 to 5 mm in diameter (fig. 1). As many as 200 colonies can be counted in a petri dish after scribing appropriately spaced parallel lines on the back of the petri dishes with a sharp point and inspecting the inverted plates on a dark-colored velvet cloth, which serves to increase contrast under fluorescent light. Plates containing more than 20 and fewer than 200 egg-yolk-positive colonies give the most reproducible counts. A few hours after the plates are ready to read, the quality of the zones begins to deteriorate, and the plates become more difficult to read.

Preparation of EYA medium

The EYA medium consists of a minimal glucose-yeast-extract agar containing 2.5% fresh egg yolk and 0.5% trisodium citrate as recommended by Donovan (1958). The minimal medium, similar to one discussed by Stanier and others (1976), consists of 1.0 g K_2HPO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.01 g $Fe_2SO_4 \cdot 7H_2O$, 0.01 g $CaCl_2$, 1.0 g glucose (Difco), 1.0 g NH_4Cl, 0.1 g yeast extract (Difco), and 15.0 g agar (Difco) per liter of deionized water, with the pH of the suspension adjusted to 7.2. Because of the progressive development of acidity, the medium should be prepared immediately prior to use. The agar medium is boiled on a stirring hotplate at medium temperature until clear, and 200-ml portions are distributed to 250-ml Wheaton-Kimbler culture media bottles. With Teflon-lined caps set on loosely, the media bottles are sterilized at 121°C for 15 minutes preferably in an autoclave with a stepwise exhaust. Alternatively, a pressure cooker or fast-exhaust autoclave can be used but should be allowed to cool gradually, rather than fast exhaust, to prevent excessive liquid loss. After 10 to 15 minutes of initial cooling, media bottles are further cooled to 47°C in a circulating water bath. Two-milliliter portions of 50% trisodium citrate solution (previously sterilized by autoclaving) are aseptically added to each 200 ml of medium using a 5- or 10-ml sterile disposable pipette. Large Grade B eggs are surface sterilized with 70% ethanol, carefully broken into a sterile ceramic egg yolker, and the yolk transferred to a new petri dish. After transferring 5 ml portions of egg yolk to each media bottle with disposable pipettes, the medium is thoroughly mixed by gently swirling the bottle. Care should be taken not to introduce air bubbles into the medium.

Precision

The precision of the method was estimated by doing forty replicate platings on the second 1/10 dilution of Poorman Creek and Cotter Basin soil samples from sites P-23 and C-23, respectively. The inherent precision of the plating technique is shown in figure 2. For P-23, colony counts varied between 112 and 160, with a mean count of 136.5, equivalent to 13,650 spores per gram, with a relative standard deviation of 10.31%. For C-23, counts varied between 64 and 103, with a mean count of 85.4, equivalent to 8,540
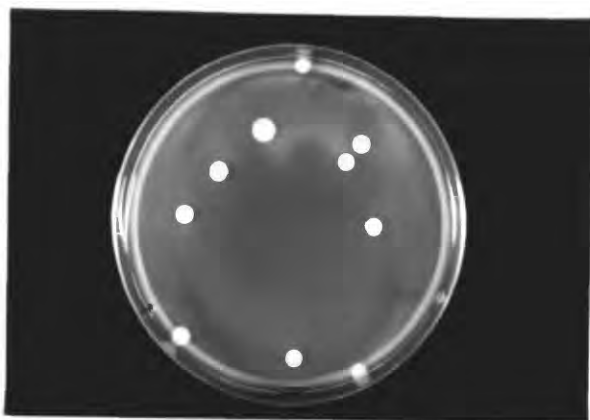
Figure 1. Typical egg-yolk reaction in a soil suspension pour plate after 15 hours of incubation at 30°C. Each zone shown on the photograph contains a small colony at the center of the zone (the colonies cannot be seen in the photograph).
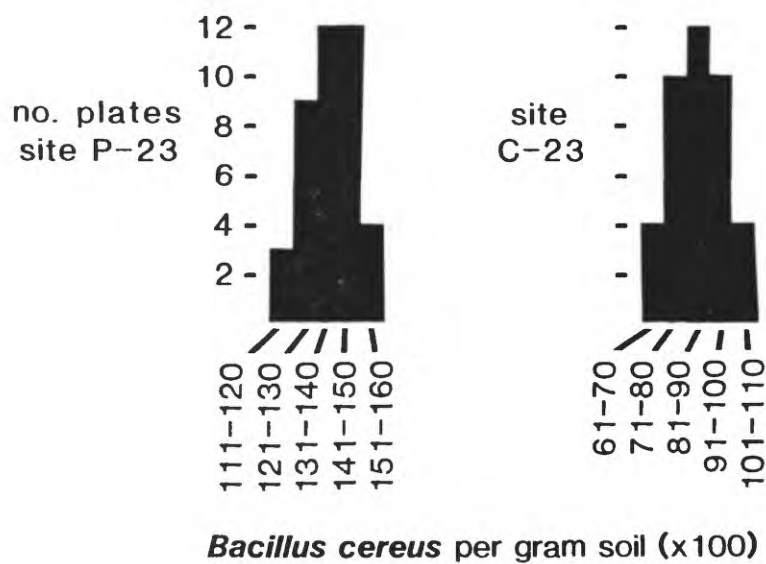
Figure 2. Distribution of <u>Bacillus cereus</u> counts obtained in forty replicate
determinations of suspensions prepared from soil sites P-23 and C-23.

spores per gram, with a similar relative standard deviation of 10.26%. As can be seen in Fig. 2, distribution is Gaussian. Standard deviations compare favorably with those obtainable with most geochemical methods in current use. The precision obtained also compares favorably with the ±25% value generally considered acceptable for replicate pour or spread plate determinations. Several pentuplicate determinations of samples with high, low, and intermediate counts from different environments have varied in about the same range as the two test samples. Considering that B. cereus counts commonly vary by as much as four and five orders of magnitude in a given study area, the test should yield quite reproducable patterns.

Specificity of the egg-yolk reaction

Of 80 strains of B. cereus tested by McGaughey and Chu (1948), all 80 were egg-yolk positive. McGaughey and Chu (1948) considered this test nearly unique among available biochemical tests in its ability to distinguish a limited number of closely related strains among the large and diverse Bacillus group. However, because a positive test may also be given by certain strains of the closely related insect pathogen, B. thuringiensis (Krieg, 1981), and more weakly by some strains of B. anthracis (McGaughey and Chu, 1948; Norris and others, 1981), the organisms so identified should, in the absence of further serological or biochemical tests, be designated presumptive B. cereus. It would appear, however, based on the much weaker egg-yolk reaction of B. anthracis (McGauhey and Chu, 1948), and what little is known about the distribution and occurrence of B. thuringiensis and its predominantly patho- genic habit (Krieg, 1981), that neither organism would significantly dilute counts of authentic B. cereus spores obtained in most North American non- agricultural soils. The data of Mishustin and Mirsoeva (1968) and Holding and others (1965), as well as summaries by DeBarjac (1981), Gordon (1981), Krieg (1981), and Norris and others (1981) do not disagree with this conclusion.

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