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Rapid and Sensitive Detection of Bacteria by Gas Chromatography

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A gas chromatograph fitted with electron capture and flame ionization detectors was employed for the rapid detection of bacteria by analysis for their metabolic products. The presence of *Proteus vulgaris*, *Streptococcus faecalis*, *S. liquefaciens*, *Escherichia coli* B, *Bacillus cereus*, and *B. popilliae* was detected in 2 to 4 hr in media inoculated with less than 10^4 cells per ml, whereas a 7- to 12-hr growth period was required for the detection of products formed in cultures of *Serratia marcescens*, *Aerobacter aerogenes*, *E. coli* K-12, *Staphylococcus aureus*, and *Salmonella typhimurium*. Metabolites elaborated by the equivalent of less than a single cell of *B. cereus*, *S. faecalis*, *P. vulgaris*, or *E. coli* B were sensed by the electron capture detector. The flame ionization detector was generally not as sensitive. Volatile metabolites were identified, and their concentrations were determined.

Methods for the rapid detection of small numbers of bacterial cells or for the sensing of minute amounts of microbial metabolites have potential applications in many aspects of microbiology and biotechnology. Highly sensitive techniques exist for the detection of some microbial metabolites which have an effect upon a suitable biological sensor system. Thus, the presence of low levels of microbiologically synthesized animal toxins, antibiotics, vitamins, and odoriferous compounds like butyric acid can be determined either quantitatively or qualitatively. However, bioassay procedures are useful for only a few compounds generated by a small group among the vast spectrum of microbial species, many are difficult to perform, and often the requisite procedures are time-consuming and require large bacterial populations to generate sufficient compound to allow for accurate assays.

In a recent study (4), it was observed that distinct patterns of volatile metabolic products characterized the various bacteria examined by means of a gas chromatograph fitted with an electron capture detector (ECD) and a flame ionization detector (FID). Because several of the substances excreted by these bacteria were detected in highly dilute solutions by ECD, an investigation was conducted to determine the sensitivity levels of this detector to several typical microbial metabolites, and it was shown that concentrations in the nanogram and occasionally the picogram range would yield a significant response in the instrument (5). Consequently, the present study was initiated to determine whether ECD-gas chromatography could be used for the rapid and sensitive detection of viable bacterial cells by examination of culture media for products generated by the metabolic activities of the organisms.

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MATERIALS AND METHODS

Aerobacter aerogenes, *Escherichia coli* B, *E. coli* K-12, *Bacillus cereus*, *Staphylococcus aureus*, *Serratia marcescens*, *Proteus vulgaris*, and *Salmonella typhimurium* were maintained on nutrient agar slants. A medium containing 4% tryptone, 1.5% yeast extract, 2% glucose, 0.6% K_2HPO_4, and 1.5% agar was used for *B. popilliae*, *Streptococcus faecalis*, *S. liquefaciens*, and *Lactobacillus bulgaricus*. After a 24-hr growth period, the bacterial cells were washed off the slants with 5.0 ml of sterile water. The cell suspension was diluted, and 0.10 ml containing 4.0 to 810 $\times$ 10^3 cells was transferred into test tubes containing 10 ml of Proom and Knight's (8) medium supplemented with 0.1% peptone. Except for *B. popilliae*, which was incubated on a rotary shaker at 30 C, the cultures were incubated without shaking at 37 C for *S. typhimurium*, *S. faecalis*, *S. liquefaciens*, and *L. bulgaricus* and at 30 C for the other species.

Replicate samples, withdrawn from the cultures at 0, 2, 4, 8, 12, 24, 48, 72, and 192 hr, were plated for the determination of viable counts. Additional samples were adjusted to *p*H 2.0 by the addition of 5 N HCl and 0.2 M HCl-KCl buffer, *p*H 2.0, and the solution was centrifuged at 2,000 $\times$ *g* for 15 min. A portion (5 ml) of the supernatant solution was saturated with anhydrous Na_2SO_4 and extracted three

times with 10-ml portions of ether. The combined ether layer was adjusted to *p*H 2.0 with 5 N HCl and concentrated to 5.0 ml in a rotary evapomix (Buchler Instruments, Inc., Fort Lee, N.J.) at room temperature. These samples were then saturated with anhydrous Na_2SO_4 for subsequent gas chromatographic analysis. Solutions containing authentic chemical compounds, samples of the water, and of the uninoculated media were treated in the same manner.

A dual-channel gas chromatograph, Aerograph model 200 (Wilkens Instrument and Research, Inc., Walnut Creek, Calif.), fitted with an FID and an ECD, was employed. The conditions for gas chromatography were essentially those described by Henis, Gould, and Alexander (4), except that the operating temperatures for the column, detectors, and injector were 100, 120, and 150 C, respectively. A 3.0-μ liter sample containing the test substance was injected into the instrument, each detector thereby receiving 1.5 μ liters.

The bacterial metabolites were identified by comparing their retention times and ϕ values (ratio of the area of the ECD peak to the area of FID peak) with those of the authentic compounds. The sensitivity limit was defined as the quantity of a metabolite or the number of viable bacterial cells required to give a peak of 10 mm² area at range 1 and attenuation 1 of the instrument.

RESULTS

Sensitivity and rapidity of detection. Upon examination of gas chromatograms of the culture extracts, it was immediately apparent that the detectors were capable of recording the presence of products elaborated by surprisingly few cells. Moreover, short incubation periods were usually adequate for the generation of sufficient metabolite to allow detection. The detection time was considered to be the time of the first sample containing peaks sensed by the particular detector which were not present in the uninoculated medium. The sensitivity and the rapidity of detection by FID of metabolites produced by 12 bacterial cultures are given in Table 1. Compounds generated by *P. vulgaris* and *L. bulgaricus* were detectable after a 2-hr incubation period, whereas a 24- to 48-hr growth period was required in *B. cereus*, *B. popilliae*, and *S. aureus* cultures for the product concentration to become sufficiently high for detection.

The sensitivity of FID for detecting bacteria, based upon sensing the presence of microbial products, was highest for *E. coli* B, metabolites formed by the equivalent of about two cells being recorded, and was lowest for *S. aureus*. These calculated values for sensitivity were obtained by dividing the number of bacteria in the 1.5-μliter sample taken at the time of detection by one-tenth the area, in mm², of the first peak found in the inoculated medium which was not present in the uninoculated solution. The data show good sensitivities for the detection of products elaborated by some species, but the sensitivities are low with other organisms.

The responses were quite different when the effluents from the column were examined with ECD (Table 2). It was immediately evident that this detector responded to at least some metabolites with greater sensitivity than did FID; hence, the presence of fewer cells in the medium could be detected, and the requisite incubation period was consequently shortened. Thus, products elaborated by as few as 0.2 to 2 cells of *B. cereus*, *S. faecalis*, *P. vulgaris*, *E. coli* B, and *B. popilliae* yielded discernible peaks. However, ECD was relatively less sensitive for metabolites of *S.*

TABLE 1. *Detection of viable bacteria by means of the flame ionization detector*

Organism	Bacteria/ml × 10^8		Detection time	Area of differential peak	Sensitivity[a]
	0 hr	At time of detection			
			hr	*mm²*	
Aerobacter aerogenes	1.7	44,000	12	886	745
Escherichia coli B	1.0	5.7	4	46	1.8
E. coli K-12	5.3	850	7	575	22.2
Bacillus cereus	0.4	1,900,000	24	1,952	1,490
B. popilliae	4.5	8,700	24	210	620
Staphylococcus aureus	4.9	5,000,000	48	520	110,000
Streptococcus faecalis	3.4	140	4	66	31.8
S. liquefaciens	8.3	200	4	400	7.5
Lactobacillus bulgaricus	81	120	2	45	40
Serratia marcescens	9.2	1,800	7	13	2,000
Proteus vulgaris	1.8	4.3	2	12	5.4
Salmonella typhimurium	8.6	220,000	12	702	4,700

[a] Bacteria/10 mm² peak.

TABLE 2. *Detection of viable bacteria by means of the electron capture detector*

Organism	Bacteria/ml × 10^3		Detection time	Area of differential peak	Sensitivity
	0 hr	At time of detection			
			hr	*mm²*	
Aerobacter aerogenes	1.7	44,000	12	3,110	210
Escherichia coli B	1.0	5.7	4	128	0.67
E. coli K-12	5.3	120,000	12	338	5,300
Bacillus cereus	0.4	2.0	4	176	0.17
B. popilliae	4.5	120	4	904	2.0
Staphylococcus aureus	4.9	5,800	12	960	91
Streptococcus faecalis	3.4	7.3	2	576	0.19
S. liquefaciens	8.3	29,000	7	840	520
Lactobacillus bulgaricus	81	1,000	7	25	600
Serratia marcescens	9.2	1,800	7	48	560
Proteus vulgaris	1.8	4.3	2	320	0.20
Salmonella typhimurium	8.6	220,000	12	244	13,000

typhimurium, *E. coli* K-12, *L. bulgaricus*, and *S. marcescens*.

By contrast with FID, the time required for the detection of most organisms with ECD was less than 12 hr. With *S. faecalis* and *P. vulgaris*, the areas of the peaks found at the first sampling time (2 hr) were sufficiently large to indicate that the presence of the organism could have been observed earlier. On the other hand, many cells were present in the broth and longer growth periods were required when *E. coli* K-12 or *S. typhimurium* was the test organism.

Bacterial signatures. Chromatographic signatures of each of the bacteria were obtained by assigning letters to the peaks in order of their retention times, as described by Henis et al. (4). The signature of each of these cultures was different, whether the signatures were obtained from FID or ECD chromatograms (Table 3). Significant distinctions were found even between the two *E. coli* strains. A larger number of peaks appeared in ECD than in FID chromatograms so that the former detector was advantageous in this regard, as well as in terms of sensitivity. However, the FID is a useful adjunct in establishing gas chromatographic signatures because it may permit the differentiation of cultures with similar or identical ECD signatures, as for example, *E. coli* B and *S. marcescens*.

Characterization of metabolites. To identify the products yielding peaks in the gas chromatograms, the retention times and ϕ values of the unknowns and of several compounds known to be produced by bacteria were compared. Identical chromatographic conditions were employed for the unknown and authentic compounds, and the concentration of the metabolites was determined from standard curves, based upon peak areas,

TABLE 3. *Flame ionization detector and electron capture detector signatures of various bacteria in growing cultures*

Organism	Signatures[a]	
	FID	ECD
Aerobacter aerogenes	CFIJKLM	BCDEFJKLM
Escherichia coli B	DHK	CDEFGH
E. coli K-12	DGL	CDEFGHKQ
Bacillus cereus	AB	CDFG
B. popilliae	CGK	CDEFGHJLP
Staphylococcus aureus	EFGIJL	CDEFGIKLMN
Streptococcus faecalis	CL	CDEFGI
S. liquefaciens	CDF	CDFIJL
Lactobacillus bulgaricus	BFGIK	BCEFGHI
Serratia marcescens	BFM	CDFGH
Proteus vulgaris	DO	CDEFGIK
Salmonella typhimurium	BC	EFGK

[a] The respective retention times for peaks A through Q are 25, 40, 50, 60, 65, 80, 105, 115, 125, 150, 160, 185, 210, 225, 250, 305, and 626 sec.

established after the microbial products were identified.

The product yields in samples taken at 2, 4, 8, 12, 24, 48, 72, and 192 hr were determined quantitatively by ECD. Six products were readily identifiable, namely, *n*-propanol, *n*-butyl alcohol, crotonic acid, acetoin, propionic acid, and isobutyric acid, with retention times of 40, 80, 110, 150, 680, and 765 sec, respectively. Some of the bacteria excreted one or more of these compounds during the active phase of growth and then destroyed them, so little or none remained in the broth at the end of the incubation period. Other organisms excreted certain products only after the termination of active growth, and the compounds remained in the medium at the last sampling

TABLE 4. *Maximal concentration of metabolites in growing cultures*

Organism	Acetoin		*n*-Propanol		*n*-Butyl alcohol		Crotonic acid		Propionic acid		Isobutyric acid	
	Time	Concn	Time	Concn	Time	Concn	Time	Concn	Time	Concn	Time	Concn
	hr	*ng/ml*	*hr*	*μg/ml*	*hr*	*mg/ml*	*hr*	*μg/ml*	*hr*	*mg/ml*	*hr*	*mg/ml*
Aerobacter aerogenes	24	3,300	72	590	48	19.2	—	0	—	0	—	0
Escherichia coli B	48	149	24	530	48	7.1	8	690	—	0	—	0
E. coli K-12	24	170	24	270	—	—	72	113	—	0	—	0
Bacillus cereus	72	690	192	2,700	48	29.0	192	400	72	7.0	—	0
B. popilliae	24	93	8	400	—	0	—	0	—	0	—	0
Staphylococcus aureus	48	144	—	0	—	0	48	320	—	0	—	0
Streptococcus faecalis	12	213	—	0	—	0	—	0	—	0	—	0
S. liquefaciens	8	22	—	0	4	32.0	—	0	—	0	—	0
Lactobacillus bulgaricus	192	9	12	760	—	0	72	69	—	0	—	0
Serratia marcescens	24	16	72	340	8	2.7	—	0	24	3.5	—	0
Proteus vulgaris	—	0	72	600	—	0	—	0	—	0	192	9.4
Salmonella typhimurium	—	0	24	300	—	—	—	0	—	0	—	0

period. Because of the large volume of data obtained, only the maximal yield and the time at which the maximum was observed are recorded. It should be pointed out, however, that metabolite levels below the sensitivities of conventional chemical procedures were detected, a fact made apparent by the extreme responsiveness of ECD (5).

It was possible by ECD-gas chromatography to demonstrate that many bacteria were capable of forming acetoin, even an organism like *L. bulgaricus*, in which the maximal yield was only 9 ng/ml (Table 4). *n*-Propanol and *n*-butyl alcohol were produced by many of the cultures, but these compounds were commonly present in much higher concentrations than acetoin. Crotonic acid formation was also reasonably common to the test bacteria, whereas detectable quantities of propionic and isobutyric acids were generated in the test medium by only an occasional culture.

DISCUSSION

We have previously demonstrated, with authentic chemicals, the extreme responsiveness of ECD to certain typical microbial metabolites (5). By applying techniques employing this remarkably sensitive detector to bacteria proliferating in a normal growth medium, it was possible to detect the activity associated with very small numbers of cells. It should be pointed out, however, that the sensitivities herein listed are based upon responses of the instrument to metabolic products rather than to cells themselves and that the sensitivities vary markedly from species to species and even, as indicated by the data for *E. coli*, from strain to strain. The sensitivities were not expressed as numbers of cells per milliliter, as is common in bacteriology, but were expressed in terms of the response of the detector to products formed by the cells present in a far smaller sample of the culture medium. However, larger samples of the culture could be used and the products could be concentrated prior to chromatography.

Because of the responsiveness of ECD, the presence of bacteria at very low population densities can be established rapidly. In some instances, a sizable peak appeared in the chromatograms at the first sampling period, 2 hr after inoculation, at which time the numbers of cells were either slightly less or somewhat more than double that present at the time of inoculation. Hence, although the compounds observed as peaks in the chromatograms generally represent substances formed by actively growing cultures, it is possible that in some instances the observed metabolites are generated by cultures still in the lag phase.

Gas chromatographic techniques have been used in many studies to differentiate selected microbial species either by examining the cells or extracellular products directly (1, 2, 4, 6, 7) or following pyrolysis (3, 9). In some investigations, individual classes of compounds were examined, whereas in others no comparable systematic approach was attempted. The identities of many of the products recorded on the chromatograms are known, but many are not. Although it is still not clear which microbial groups may be amenable to biochemical taxonomy employing chromatographic methods or even which types of

microbial metabolites should be examined, the fact that ultrasensitive chromatographic techniques are available opens the door to the possibility that not only can the presence of certain microorganisms in low cell numbers be detected but also that the organisms may be differentiated into at least broad taxonomic categories. Likewise, the existence of as yet unknown metabolites may be revealed by these chromatographic methods—compounds which have been overlooked because of their low yield and the lack of a suitable biological or nonbiological sensor.

Other applications are suggested by the sensitivity to microbial cells or, more properly, microbial products. For example, it should be feasible to establish the sensitivity of pathogens to antibiotics far more rapidly than by existing methods, because only a short incubation period is required, even with small inocula, to allow for sufficient product to be elaborated to induce a response in the detector. Similarly, in a living organism or in a natural or manufactured material in which low levels of microbial contamination are anticipated or in which it is desired to determine whether microbial activity is becoming prominent, ultrasensitive detectors with gas chromatography may be of value. Thus, ECD might be used for the demonstration of the presence or activity of pathogenic agents in the body of their hosts by detecting signs of physiological stress or minute quantities of specific metabolites produced in the early stages of infection. Recently, we have observed chromatographically discernible biochemical changes prior to the appearance of any clinical symptoms in animals infected with equine infectious anemia virus (Mitruka and Alexander, *unpublished data*). Other potential applications of this technique are not difficult to find.

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