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Note

Studies on volatile metabolites of some potentially pathogenic *Bacillus* species, using automated head-space gas chromatography

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Use of head-space gas chromatography (HSGC) has now been extended to diagnostic microbiology. HSGC enables the detection of volatile bacterial metabolites. The technique is simple and, because of its suitability for automation, it has high sample capacity. Automated HSGC, with capillary column, has been utilized in the identification of anaerobic bacteria [1] and for rapid diagnosis of bacteraemia [2].

Several species of *Bacillus* have recently been claimed as causal agents in a variety of pathologic conditions, e.g. abscesses, pneumonia, osteomyelitis, bacteraemia, endocarditis, bovine mastitis and food-poisoning [3]. The recognition of this biochemically heterogeneous group of bacteria is therefore increasingly important. The present investigation was undertaken to evaluate the differential diagnostic capacity of HSGC in regard to some potentially pathogenic species of *Bacillus* by analysis of volatile acidic and neutral metabolites.

EXPERIMENTAL

The study comprised 65 strains, representing altogether seventeen species of *Bacillus* (Table I). All strains were subcultured on blood agar plates and incubated under aerobic conditions in 100-ml flasks containing 20 ml of

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TABLE I

STRAINS OF *BACILLUS* SPECIES INVESTIGATED

Organism	No. of strains from clinical specimens	Reference strains	Total No. of strains
B. alvei	—	NCTC 6352, NCTC 3349	2
B. badius	—	NCTC 10333	1
B. brevis	—	NCTC 2611	1
B. cereus	10	NCTC 2599, NCTC 6474	12
B. circulans	3	NCTC 5846, NCTC 2610	5
B. coagulans	3	NCTC 3991, NCTC 10334	5
B firmus	1	NCTC 10335, CCM 2212	3
B. laterosporus	—	NCTC 6357, NCTC 2613	2
B lichteniformis	5	NCTC 10341, NCTC 962	7
B macerans	1	NCTC 6355, NCTC 1068	3
B. megaterium	—	NCTC 10342, NCTC 5635	2
B pantothenticus	1	NCTC 8122, NCTC 8162	3
B. polymyxa	3	NCTC 10343, NCTC 4744	5
B pumilus	—	NCTC 10337, NCTC 2595	2
B sphaericus	1	NCTC 10338, NCTC 2608	3
B. stearothermophilus	3	NCTC 10339	4
B. subtilis	3	NCTC 3610, NCTC 5398	5
Total	34	31	65

trypticase soy broth (TSB) at 30°C, all cultures in duplicate, for 24 and 48 h respectively. Strains of *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Shigella sonnei*, *Staphylococcus aureus*, *Streptococcus faecalis* and *Yersinia enterocolitica*, all isolated from clinical specimens at the Bacteriology Department, University Hospital, Lund, were incubated at 37°C but otherwise under identical conditions as the *Bacillus* strains. For evaluation of reproducibility, *B. cereus* (NCTC 2599) and *B. macerans* (NCTC 6355) were additionally each incubated in five flasks with TSB for 24 h after which 2-methylpentanoic acid was added as internal standard to a final concentration of 0.005% (w/w). The reference mixture in HSGC analyses was an aqueous solution of acetic (16 m*M*), propionic (6 m*M*), isobutyric (2 m*M*), butyric (4 m*M*), isovaleric (1 m*M*) and valeric (1 m*M*) acids. From the broth cultures, from non-inoculated TSB medium and from the reference solution, 1-ml aliquots were transferred to glass ampoules fitting into the automatic turntable of the HSGC analyzer. To each ampoule were then added five drops of sulphuric acid (25%, w/w, in water) and a saturating amount of solid magnesium sulphate. The ampoules were thereafter sealed with PTFE-lined rubber membranes and aluminium crimp caps.

The gas chromatograph (F 45, Perkin-Elmer) was equipped with a unit for automatic head-space injection, a flame ionization detector at 150°C and a 25-m fused-silica capillary column coated with SP-1000. No attenuation of the detector signal was used. Before the analysis the samples were heated in the automatic turntable of the instrument at 80°C for at least 20 min to ensure temperature equilibration. The flow-rate of the carrier gas (nitrogen) was

0.8 ml/min at a split ratio of 1:12. The column was held at 110°C, the injection needle at 250°C and the injector at 200°C.

RESULTS AND DISCUSSION

With this HSGC system, all the fatty acids in the standard solution could readily be separated within 8 min. Sterile TSB medium incubated under exactly the same conditions as medium inoculated with the *Bacillus* species gave chromatograms which contained few and negligibly small peaks in comparison with those obtained in analyses of broth cultures (Fig. 1).

Chromatograms representing *Bacillus badius*, *B. brevis*, *B. cereus*, *B. circulans*, *B. coagulans*, *B. lichteniformis*, *B. megaterium*, *B. polymyxa*, *B. pumilus*, *B. sphaericus*, *B. stearothermophilus* and *B. subtilis* were all very much alike. After 24 h of incubation the most prominent peaks corresponded to ethanol, and acetic, isobutyric and isovaleric acids. There were also minor yields of propionic acid and butyric acid. *B. firmus* and *B. laterosporus* gave somewhat lower yields of the same fatty acids. Apart from acetic acid, *B. alvei*, *B. macerans* and *B. pantothenticus* produced no detectable amounts of acids even after 48 h of incubation, although heavy growth was observed. The major peak in these chromatograms instead corresponded to ethanol. Typical chromatograms of *B. cereus* and *B. macerans* with the internal standard (2-methylpentanoic acid) added, are shown in Fig. 1. For *B. cereus*, the peak areas related to the internal standard at the five reproducibility studies were in the range 0.20—0.32 (acetic acid), 0.09—0.11 (propionic acid), 0.92—1.20 (isobutyric acid), 0.10—0.14 (butyric acid) and 1.08—1.42 (isovaleric acid). None of the organisms other than *Bacillus* — all of which are common food-poisoning agents — produced more than trace amounts of isobutyric and isovaleric acids.

In addition to *B. anthracis*, several species of *Bacillus* are now recognized as causing a variety of severe infections [4, 5]. *B. cereus*, for example, is considered to be a food-poisoning agent [6] and can cause panophthalmia. Further, *B. subtilis*, *B. lichteniformis* and, though more rarely, also other *Bacillus* species, are occasionally found as clinical or food-borne pathogens [7]. Despite the published reports, the pathogenicity of these organisms has been slow in gaining general acceptance [3].

Bacteria of the genus *Bacillus* are characterized as aerobic spore-formers of gram-type positive, negative or zero [8]. Current taxonomic designations are based on a variety of analytical procedures, including conventional biochemical tests [9, 10], enumerative, sometimes computerized, techniques [11—14], DNA homology studies [15], serology [16] and chromatography [17, 18]. With these procedures, singly or in certain combinations, it is possible to demonstrate differences within and between species. That several *Bacillus* species produce appreciable amounts of isobutyric and isovaleric acids, as demonstrated in our study, does not seem to have been reported previously.

The chromatographic results indicated unity rather than differentiation of the studied species, and thus metabolic homogeneity. Of the seventeen *Bacillus* species studied, fourteen gave virtually identical chromatographic patterns, which could be easily distinguished from those of several other organisms which commonly occur as food-poisoning agents. Gas chromatography appears

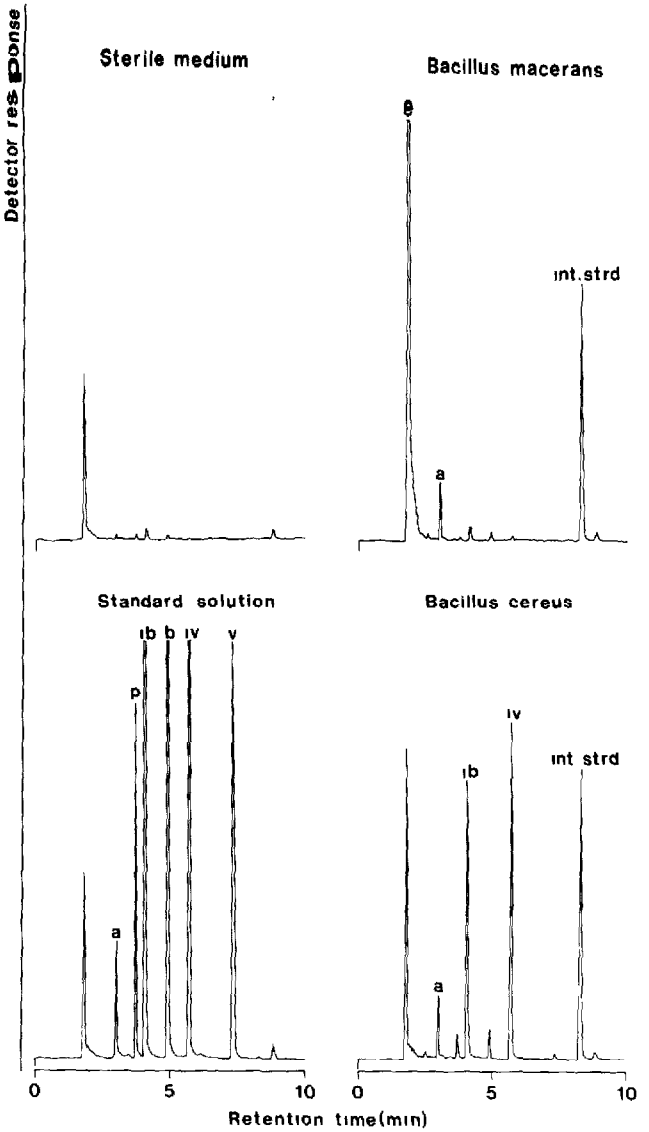

Fig. 1. Chromatograms (head-space gas chromatography) obtained from analyses of a standard solution of fatty acids, of non-inoculated trypticase soy broth medium, and of the same medium incubated for 24 h with *B. cereus* or *B. macerans*. e = Ethanol, a = acetic acid, p = propionic acid, ib = isobutyric acid, b = butyric acid, iv = isovaleric acid, v = valeric acid; int strd. (Internal standard) = 2-methylpentanoic acid. See text for specification of test conditions.

to be a useful tool in epidemiologic studies when the presence of *Bacillus* is suspected.

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