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CHARACTERIZATION OF PATHOGENIC BACTERIA BY AUTOMATED HEADSPACE CONCENTRATION—GAS CHROMATOGRAPHY

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SUMMARY

Automated headspace concentration—gas chromatography (AHC—GC) was used to profile the volatile metabolites produced by *Proteus mirabilis*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Bacterial cultures were incubated in trypticase soy broth and examined at 24 h. The profiles were consistent for each genus examined and variation observed among the different strains of each species was chiefly quantitative. The volatiles were identified by concurrent headspace concentration- gas chromatography—mass spectrometry and consisted mainly of isobutanol, isopentanol, isopentyl acetate, 1-undecene and methyl ketones. There were sufficient differences in the profiles in the 4—6 min elution period to distinguish *P. aeruginosa* and *S. aureus* from each other and from the other two bacteria. *P. mirabilis* and *K. pneumoniae* typically showed three intense peaks which corresponded to isobutanol, isopentyl acetate and isopentanol. The determination of volatiles by AHC—GC is sensitive, rapid and offers a possible alternative for automatic detection and characterization of pathogenic bacteria.

INTRODUCTION

A variety of gas chromatographic (GC) techniques have been applied to the study of microbial cell metabolites and components. The object of such analytical studies has been to obtain information useful for the rapid identification of microbial genera or species. In whole-cell analyses the thermally degraded products of isolated microorganisms have yielded pyrolysis—gas chromatographic profiles that are genus-, species- and even strain-specific [1]. More commonly, bacteria have been analyzed by GC after the hydrolysis and

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extraction of cell components. Profiles of cellular fatty acid esters have been obtained for many microbial groups and are widely used in bacterial taxonomy, especially among organisms such as the corynebacteria, which are often unreactive in conventional bacteriological tests [2, 3]. Cellular carbohydrates and hydroxy acids have also proven useful in microbial taxonomy [4, 5].

Bacterial metabolite analysis by GC does not require isolated organisms and is generally faster and easier to perform than cell component analysis. Metabolites from culture fluids can be isolated by solvent extraction or even analysed by direct aqueous injection. Volatile metabolites can be rapidly determined by headspace analysis. Direct headspace analysis and headspace concentration have shown that bacteria produce acids, alcohols, ketones, amines, sulfides and hydrocarbons useful for taxonomy and identification [6–9]. However, manual procedures for headspace concentration and sample transfer have the potential for giving variable results. In automated headspace concentration–gas chromatography (AHC–GC) the entire analytical sequence from the collection of volatiles to GC analysis is totally automated yielding more consistent results. In the following study utilizing AHC–GC we have examined the volatiles produced by four pathogenic bacteria, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Proteus mirabilis* and *Klebsiella pneumoniae*, and report the production of headspace profiles sufficient to distinguish these bacterial genera.

EXPERIMENTAL

Bacterial cultures

Microorganisms included in this study were strains of *P. aeruginosa* [6], *K. pneumoniae* [6], *P. mirabilis* [4] and *S. aureus* [4]. Clinical isolates from the axilla, foot, nares, hand and perianal regions were obtained from Duhring Labs., Department of Dermatology, University of Pennsylvania Medical School and the Pennsylvania College of Podiatric Medicine. These were identified by the API test system (Analytab Products). Also included were strains *P. aeruginosa* ATCC 19660 and CDC 9171, *K. pneumoniae* ATCC 13906 and ATCC E13883 and *S. aureus* ATCC 21684. The organisms were inoculated on slants of nutrient agar (Difco Labs., Midland, MI, U.S.A.) containing 0.1% glucose and 0.1% yeast and incubated for 24 h at 37°C. The growth was suspended in 3 ml sterile distilled water and 1 ml was used to inoculate 50 ml sterile trypticase soy broth (TSB) (BBL Microbiology Systems, Cockeysville, MD, U.S.A.) in a 250-ml Erlenmeyer flask with a 24/40 standard taper joint. The flask was stoppered with cotton and incubated in a rotary water bath at 37°C for 24 h.

Analytical conditions

Headspace volatiles were analyzed on a UNACON 810B automated headspace concentrator–gas chromatograph incorporating wide- and narrow-bore adsorption traps in series and a flame ionization detector (Envirochem, Kemblesville, PA, U.S.A.) as previously described [9]. The initial trap of the instrument was packed with approx. 180 mg of 80–100 mesh Tenax GC (Applied Sciences, Bellefonte, PA, U.S.A.). A 48-mm PTFE stir bar was placed

in the culture flask which was then fitted with a one-hole PTFE plug, enclosed in a heating mantle set at 37°C and attached to the inlet of the instrument. The culture was magnetically stirred and the headspace swept with nitrogen for 20 min at a flow-rate of 60 ml/min. The trapped organics were automatically desorbed at 200–220°C and backflushed to the column for separation as in a normal GC analysis. This study employed a CPWAX-57CB chemically bonded high-capacity fused-silica capillary column (25 m × 0.33 mm with 1.3 μm film thickness) (Chrompack, Bridgewater, NJ, U.S.A.). Hydrogen was the carrier gas and the oven programme was 60°C for 4 min and 60–180°C (at 6°C/min). The detector response was recorded on an SP-4100 computing integrator (Spectra-Physics, Baltimore, MD, U.S.A.) which automatically recorded retention times, relative retention times and integrated areas. The gas chromatograph and integrator attenuations were adjusted for maximum sensitivity. [*methyl*-$^2H_3$] Anisole (200 ng) was added to each culture before analysis to monitor transfer efficiency and served as an internal standard for calculating relative retention times. The retention time for anisole was 10.34 ± 0.05 min. The retention times of 40 authentic compounds formed the data base for the identification of bacterial volatiles. A sample of 1-undecene gave five peaks of relative intensity 2.3, 1.0, 3.9, 4.4 and 2.1 at relative retention times of 0.43, 0.50, 0.51, 0.55 and 0.58 when injected onto the trap of the UNACON concentrator. The same material when injected onto the same polarity column in the gas chromatographic–mass spectrometric (GC—MS) system gave only a single peak. Subsequent use of helium as the transfer and carrier gas in the UNACON concentrator resulted in only a single peak at a relative retention time of 0.55 for 1-undecene. Similarly sulfur compounds (dimethyldisulfide) were only detectable when helium was used.

In two species from each genus peak assignments were confirmed using headspace concentration—gas chromatography–mass spectrometry (HC—GC–MS). This was performed as previously described [8] except that a Finnigan 4510 gas chromatograph–mass spectrometer was used with a CPWAX-57CB capillary column programmed at 50°C for 4 min to 220°C (at 4°C/min). A specially designed thermal tube desorber (Envirochem) was interfaced to the injector port of the gas chromatograph to provide rapid heating and ease of tube exchange. Volatiles were concentrated on Tenax-GC for 40 min and desorbed and condensed on the GC capillary column with cooling obtained by passing nitrogen through a copper coil immersed in liquid nitrogen and through a metal sleeve which enclosed the first 24 cm of the column. Both electron-impact and chemical-ionization spectra were obtained. Component identities were confirmed by computer matching with compounds in the National Bureau of Standards library.

RESULTS

Representative AHC—GC profiles produced by *P. mirabilis*, *P. aeruginosa*, *K. pneumoniae* and *S. aureus* after 24 h incubation in TSB are shown in Fig. 1. These analyses were done on the same column over a period of two years and were found to be reproducible in terms of the relative appearance of the profiles and retention times. Most bacterial volatiles considered in this study

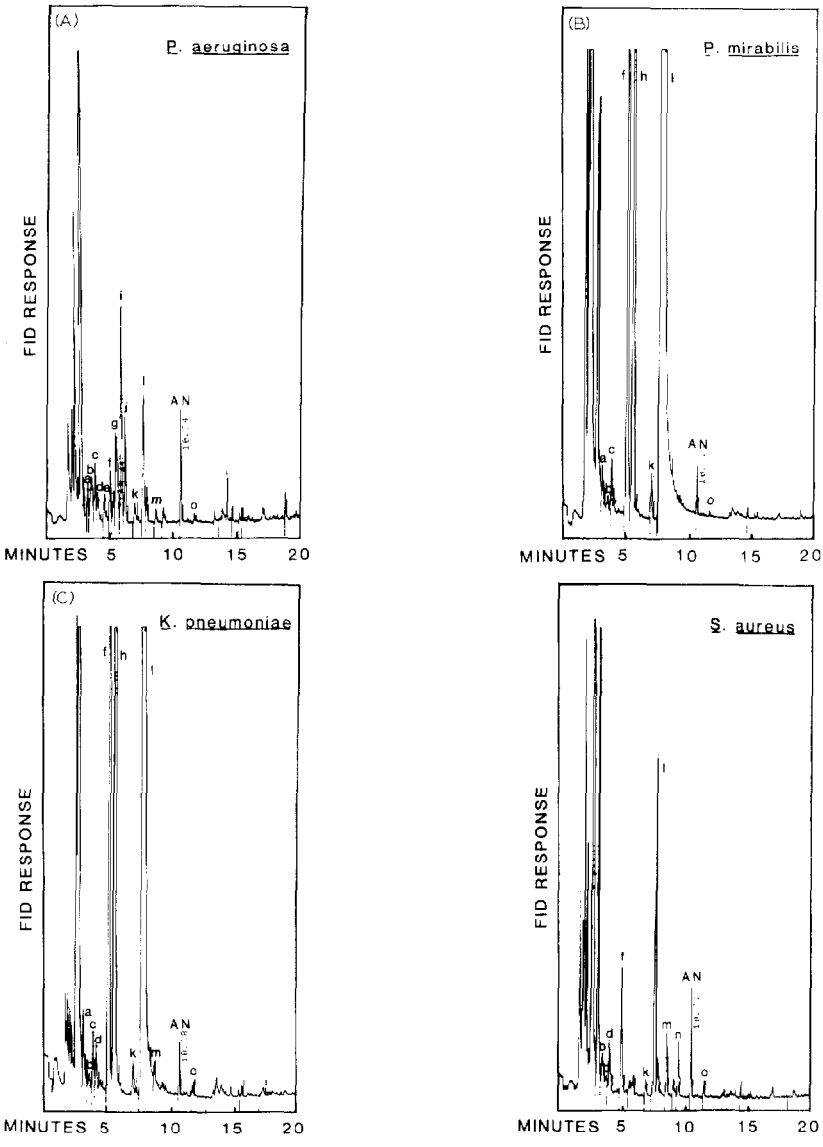

Fig. 1. Volatile metabolic profiles of *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Klebsiella pneumoniae* and *Staphylococcus aureus* with hydrogen as transfer and carrier gas. For peak identification, see Table I.

eluted between 3 and 12 min. Peaks eluting prior to 3 min were also in the background from media and were not considered in the analysis.

The bacterial headspace metabolites consisted of three to six compounds present as major peaks and the remaining volatiles as relatively minor constituents. In order to determine the frequency with which volatile metabolites occur within different strains of a given species, four to six isolates of each bacterium were analyzed in duplicate by AHC—GC. Variation existed in peak areas from analyses to analyses, but as shown in Table I the production of volatiles for a given genus and species was consistent. The prominent peaks

TABLE I

SUMMARY OF VOLATILES DETECTED IN BACTERIAL HEADSPACE

The numbers between parentheses refer to the number of strains which had this compound of six, six, four or four for the respective organisms.

Compound	RRT*	Organisms			
		K. pneumoniae	*P. aeruginosa*	*P. mirabilis*	*S. aureus*
a. Butanone	0.30	+	+		+
b. Methylbutanal	0.33	+ (3)	+	+ (2)	+
c. 2-Butanol	0.36	+	+	+	+ (1)
d. Pentanone/toluene	0.38	+	+ (3)		+
e. Undecane	0.43	+ (2)**	+		
A. Dimethyldisulfide***	0.45	+	+	+	+
f. Isobutanol	0.47	+	+	+	+
g. Undecene	0.50		+		
h. Isopentyl acetate	0.51	+	+**	+	
i. 1-Undecene	0.55		+		
j. Butanol	0.58		+		
k. 2-Heptanone	0.66	+	+	+	+
l. Isopentanol	0.74	+	+	+	+
m. Methylbutenol	0.82	+	+ (3)		+
n. Acetoin	0.88				+
o. 2-Nonanone§	1.10	+ (3)	+ (2)	+ (2)	+ (1)
p. 2-Undecanone§	1.50	+	+	+ (1)	

*Retention times relative to anisole (AN) = 1.0.
**Unknown peak of same retention time.
***With helium as transfer and carrier gas.
§Found in GC—MS studies of all organisms though present in trace amounts here.

produced by *S. aureus* were isobutanol, isopentanol and acetoin. *P. aeruginosa* headspace included a series of peaks of similar intensity, primarily isobutanol, undecene, butanol and isopentanol, with the overall profile differing from *S. aureus*. The abilities of *P. mirabilis* and *K. pneumoniae* to produce as major components isobutanol, isopentyl acetate and isopentanol were distinguishing features of these bacteria. The concentration of these peaks relative to anisole was calculated for all strains of these two species. The ratio of isopentanol to the other components was three to four times higher for *P. mirabilis*; however, the variations in peak areas between individual strains was too great to be of predictive value.

When the production of *K. pneumoniae* volatiles was studied in relationship to incubation time, the three peaks representing isobutanol, isopentyl acetate and isopentanol increased with time over a 5—24 h period. Other experiments examined the effect on headspace volatiles of enrichment of TSB with amino acids. This was done to confirm peak assignments. Cultures of *K. pneumoniae* enriched with 0.1 *M* valine showed nearly a ten-fold increase in amounts of isobutanol produced after 24 h. The addition of isoleucine produced no change in the ratio of the three peaks while leucine caused a five-fold increase in isoamylacetate and isopentanol. In one strain of *P. aeruginosa* isoleucine caused an increase in peaks at relative retention times 0.33 and 0.72 which correspond to

2-methylbutanal and 2-methylbutanol; no change occurred in the presence of leucine. However this was not characteristic of all *P. aeruginosa* strains.

A part of the complexity observed for *P. aeruginosa* is due to the reactivity of undecene in the analysis system. When hydrogen was used for transfer of volatiles to the GC column, 1-undecene was hydrogenated to undecane and isomerized to other undecenes giving a characteristic pattern (Table I and Fig. 1). With helium in place of hydrogen, a single peak of increased intensity is found for undecene (peak i) and other undecene related peaks are absent (Fig. 2). Under these conditions an additional peak for dimethyldisulfide (peak A) is found for all the organisms (compare Figs. 1 and 2 for *P. aeruginosa* and for *K. pneumonia*). This demonstrates the dependence of a headspace concentration system which involves valves and transfer lines on the nature of the gas used to transfer the volatiles from the trap to the column.

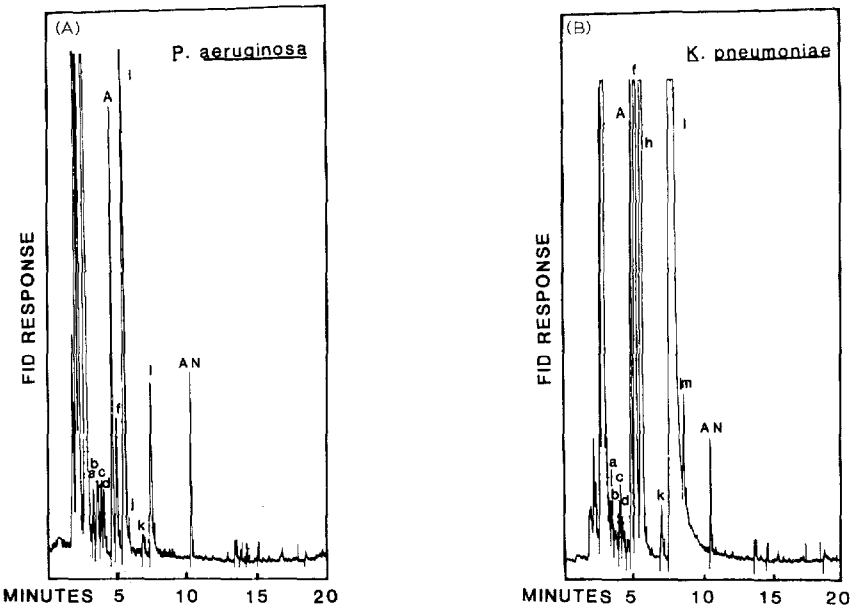

Fig. 2. Volatile metabolic profile of *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* with helium as transfer and carrier gas. For peak identification, see Table I.

HC—GC—MS analysis confirmed the identifications mentioned above and revealed additional less volatile metabolites for all of the bacterial species. All cultures showed varying levels of dimethyldisulfide (peak A), dimethyltrisulfide (peak C) and methyl ketones of odd-carbon numbers from C-3 to C-15. *P. mirabilis* could be differentiated by the presence of sulfur-containing compounds including methylthiopropanol, dipropyltrisulfide and thiomethyl benzoate. *S. aureus* showed trace levels of pyridine, indole (O) and in one sample isobutyric (G) and isovaleric acids (H) (Fig. 3). The additional components identified also in *Klebsiella* cultures were phenylethanol (L) and benzyl alcohol (J). Ethylmethyldisulfide, benzylmethylsulfide and 2-aminoacetophenone were present in *Pseudomonas* cultures. The peaks are not consistently discernible in the AHC—GC studies and were not used as distinguishing features in those profiles.

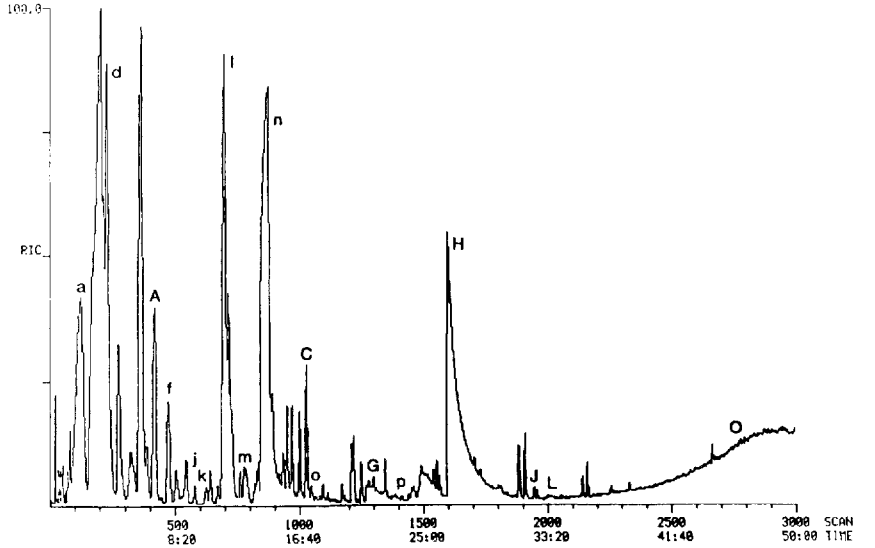

Fig. 3. Reconstructed-ion chromatogram of volatiles from *Staphylococcus aureus*. For peak identification, see Table I and text.

DISCUSSION

Analysis for microbial metabolites has promising applications as a diagnostic aid. Diseased body fluids have been examined and found to contain bacterial metabolites not present in normal fluids [10]. Metabolite analysis by GC is rapid and particularly useful when the suspected pathogen is slowly growing or difficult to isolate. For example, the GC analysis of serum arabinitol and mannose offers promise in the diagnosis of candidiasis [11, 12]. Infections caused by certain anaerobic bacteria can be diagnosed by the quantitation of acidic products in clinical specimens [13]. Headspace analysis of clinical material has detected volatile short-chain fatty acids that are indicative of anaerobic infection [6, 7]. It has also been proposed that *Escherichia coli*, *K. pneumoniae* and *P. mirabilis* in urine can be detected by analysis for ethanol or dimethyldisulfide [14–17] produced after subculture on appropriate media. Volatiles might additionally serve as indicators of bacterially spoiled foods. Alcohols, ketones, hydrocarbons, sulfides, amines and esters are present in the headspace of meats and fish contaminated with *Pseudomonas*, *Achromobacter* and *Moraxella* species [18–23].

The present study demonstrates the production of characteristic volatile metabolites by *P. mirabilis*, *K. pneumoniae*, *P. aeruginosa* and *S. aureus*. Our results confirm the presence of isobutanol and isopentanol in the headspace of *P. mirabilis* and *K. pneumoniae* [15, 24]. We additionally describe the ability of these two bacteria to produce isopentyl acetate, which we believe to be the result of a secondary reaction between acetic acid and isopentanol. Acetic acid and propionic acid have been reported to be produced by species of *Klebsiella*, *Staphylococcus* and *Proteus* [25]. Volatile production by *K. pneumoniae* was rapid and a 10–15 h incubation period was sufficient to permit the production of these characteristic volatiles. Valine enrichment enhanced isobutanol pro-

duction by *K. pneumoniae*. A similar situation was noted for *P. mirabilis* and results from sequential decarboxylation and oxidative deamination of the amino acid [15].

P. aeruginosa headspace produced a profile that mainly consisted of alcohols, methyl ketones and a characteristic peak of 1-undecene. Several studies have indicated the production of 1-undecene by pseudomonads in general and *P. aeruginosa* in particular. [8, 9, 18]. *S. aureus* produced less intense peaks than the other bacteria examined but still produced a characteristic profile of alcohols and acetoin. The greater sensitivity of headspace concentration compared to direct sampling suggests that AHC—GC can expand the number of organisms detectable by volatile metabolite analysis.

Simple colorimetric analyses for volatiles such as acetoin, indole and hydrogen sulfide constitute traditional tests for the identification of bacteria. From the present study and work of other investigators it is reasonable to conclude that other volatiles of diagnostic significance may be produced in amounts sufficient to be detected by such procedures. The ability to develop a series of reagent assays sensitive to the variety of volatiles produced by bacteria could be the end result of a thorough understanding of the volatiles produced by microorganisms.

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REFERENCES

1 C. Gutteridge and J. Norris, J. Appl. Bacteriol., 47 (1979) 5—43.
2 K. Suzuki and K. Komegata, Int. J. Syst. Bacteriol., 33 (1983) 188—200.
3 I. Bousfield, G. Smith, T. Dando and G. Hobbs, J. Gen. Microbiol., 129 (1983) 375—394.
4 C. Cummins, J. Gen. Microbiol., 28 (1962) 35—50.
5 M. Athalye, W. Noble, A. Mallet and D. Minnikin, J. Gen. Microbiol., 130 (1984) 513—519.
6 P. Mardh, L. Larsson and G. Odham, Scand. J. Infect. Dis. Suppl. 26 (1981) 14—18.
7 A. Taylor, in B. Kolb, (Editor), Applied Headspace Gas Chromatography, Heyden, Philadelphia, PA, 1980, pp. 140—154.
8 J. Labows, K. McGinley, G. Webster and J. Leyden, J. Clin. Microbiol., 12 (1980) 512—526.
9 J. Zechman and J. Labows, Can. J. Microbiol., 31 (1985) 232—237.
10 C. Edman and J. Brooks, J. Chromatogr., 274 (1983) 1—25.
11 C. Wells, M. Sirany and D. Blazevic, J. Clin. Microbiol., 18 (1983) 353—357.
12 L. Repentigny, R. Kuykendall and E. Reiss, J. Clin. Microbiol., 17 (1983) 1166—1169.
13 N. Legakis, K. Xanthopoulou, H. Ioannidou and J. Papavassiliou, Ann. Microbiol. (Inst. Pasteur), 133B (1982) 281—290.
14 N. Hayward and T. Jeavons, J. Clin. Microbiol., 6 (1977) 202—208.
15 N. Hayward, T. Jeavons, A. Nicholson and A. Thornton, J. Clin. Microbiol., 6 (1977) 187—194.

16 K. Manja and K. Rao, J. Clin. Microbiol., 17 (1983) 264—266.
17 P. Coloe, J. Clin. Pathol., 31 (1978) 365—369.
18 R. Dainty, R. Edwards and C. Hibbard, J. Appl. Bacteriol., 57 (1984) 75—81.
19 E. Bowman, L. Freeman, D. Later and M. Lee, J. Food Sci., 48 (1983) 1358—1359.
20 A. Miller, R. Scanlan, J. Lee and L. Libbey, Appl. Microbiol., 26 (1973) 18—21.
21 B. Pittard, L. Freeman, D. Later and M. Lee, Appl. Microbiol., 43 (1982) 1504—1506.
22 M. Lee, D. Smith and L. Freeman, Appl. Microbiol., 37 (1979) 85—90.
23 P. Guarino and A. Kramer, J. Food Sci., 34 (1969) 31—37.
24 L. Larrson, P. Mardh, G. Odham and M. Carlsson, J. Clin. Pathol., 35 (1982) 715—718.
25 S. Hansen, T. Perry, D. Lesk and L. Gibson, Clin. Chim. Acta, 39 (1972) 71—74.