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RAPID IDENTIFICATION OF *BACTEROIDES* SPECIES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

High-performance liquid chromatography was evaluated for the rapid identification of *Bacteroides* species of clinical interest. Each isolate was inoculated into a defined chemical medium containing primarily carbohydrates and was incubated aerobically at 37°C for 1 h. After centrifugation, the supernatants were placed on ice to stop further enzymatic reactions. Specimens were injected into an Aminex HPX-87H column in order to determine carbohydrates and acid metabolic products. Peak areas of carbohydrates for each isolate were compared with those for uninoculated medium. As the utilization indexes of raffinose, lactose and arabinose were found to be particularly significant, the patterns of carbohydrate utilization could be used for the identification of *Bacteroides* species. This method can be adapted for diagnostic laboratory use and has good potential for automated microbial identification.

INTRODUCTION

Currently, there is considerable interest in the development of microtechniques and instrumental methods for the identification of anaerobic bacteria[1]. Many new microtest systems, such as the API 20 A (API System, Montalieu Vercieu, France) and the An-IDENT (API Analytab Products, Plainview, NY, U.S.A.), have been introduced to shorten the identification time. The use of instrumental methods for anaerobic bacteriology has involved mainly gas–liquid chromatography of volatile metabolic products and cellular fatty acids[2,3].

We have evaluated the applicability of high-performance liquid chromatography (HPLC) to the rapid identification of *Bacteroides* species by analysing changes in the composition of a defined chemical medium after aerobic incubation for 1 h.

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EXPERIMENTAL

Bacterial strains

Twelve *Bacteroides* isolates were obtained from the collection of the Institute of Microbiology, University of Genoa. The following species were tested: *B. fragilis* (three strains), *B. thetaiotaomicron* (three strains), *B. ovatus* (two strains), *B. vulgatus* (two strains) and *B. distasonis* (two strains). The *Bacteroides* were grown on blood agar plates in an anaerobic chamber for 24 h at 37°C.

Defined chemical medium

A defined chemical medium was formulated containing carbohydrates (raffinose, lactose, glucose, mannose, arabinose and salicin at final concentrations of 7.5, 7.5, 5.0, 2.5, 2.5 and 0.01 mg/ml, respectively) and the same trace factors as those reported by Harpold *et al.*[4]. All the components were dissolved in 20 m*M* potassium phosphate buffer (pH 7.0) and the medium was sterilized by passage through a filter of pore size 0.20 μm.

Aerobic incubation

Each *Bacteroides* isolate was inoculated into an aliquot (1 ml) of the filtered medium to approximately the turbidity of a No. 10 McFarland standard and incubated aerobically at 37°C for 1 h. Bacterial cells were removed by centrifugation, and the supernatants were placed on ice to stop further enzymatic reactions.

HPLC analysis

Samples (20 μl) were injected into the chromatograph for analysis. A modular system (Gynkotek, Munich, F.R.G.), equipped with an Aminex HPX-87H cation-exchange column (30 cm × 7.8 mm I.D.) for carbohydrate analysis (Bio-Rad Labs., Richmond, CA, U.S.A.) was used[5]. The column was kept at 20°C and 3.5 m*M* sulphuric acid was used as the mobile phase at a flow-rate of 0.4 ml/min. The spectrophotometer (SP-4; Gynkotek) was adjusted to 196 nm and 0.04 a.u.f.s. A Model 4290 integrator (Varian, Walnut Creek, CA, U.S.A.) was employed for peak-area quantitation.

Utilization index and production index

The carbohydrate utilization index was determined according to Harpold and Wasilauskas[6]. Lyxose was employed as an internal standard, as it was not metabolized by any of the *Bacteroides* species examined. Zero utilization of a carbohydrate would give an index value of 1 and complete utilization would give 0. A value of greater than 1 could occur if a metabolic product were eluted together with one of the carbohydrates. Indeed, some new compounds that were not present in the original medium were produced during incubation. Production indexes for these new peaks were determined by dividing their areas by that of the internal standard peak.

RESULTS

The HPLC profile of the individual carbohydrates for the uninoculated medium is shown in Fig. 1. Fig. 2 shows a chromatogram of the medium after inoculation

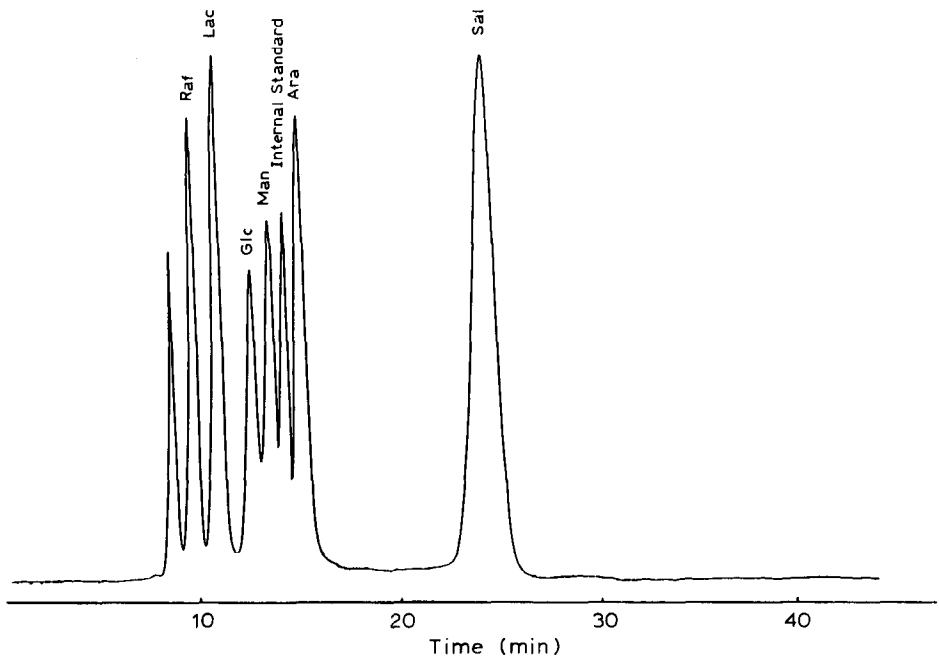

Fig. 1. HPLC profile of the defined chemical medium. Abbreviations as in Table I.

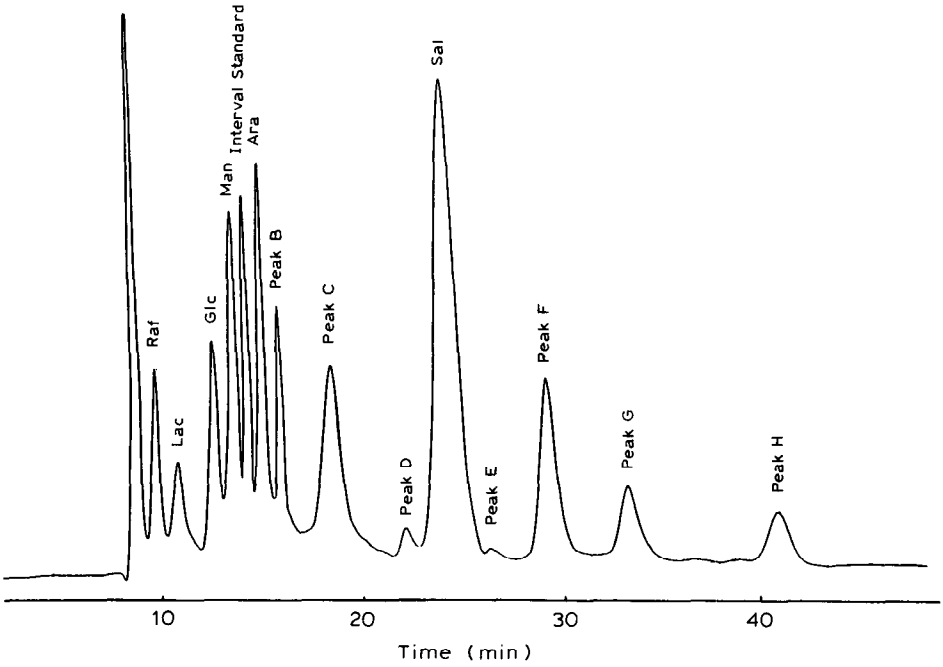

Fig. 2. HPLC profile of *B. fragilis* supernatant after aerobic incubation. Abbreviations as in Table I.

TABLE I

CARBOHYDRATE ULTILIZATION BY *BACTEROIDES*

Species	*Utilization index**					
	Raf	*Lac*	*Glc*	*Man*	*Ara*	*Sal*
B. fragilis	0.33	0.05	0.78	0.82	1.00	1.02
B. thetaiotaomicron	0.60	0.08	0.57	0.75	0.82	1.06
B. ovatus	0.85	0.75	0.65	0.85	0.58	0.78
B. vulgatus	0.12	0.52	0.82	N.D.	0.80	0.90
B. distasonis	0.90	0.84	0.74	0.78	1.02	0.82

* Data represent the mean values of the results obtained for the individual *Bacteroides* strains tested. Raf = raffinose; Lac = lactose; Glc = glucose; Man = mannose; Ara = arabinose; Sal = salicin.

with *B. fragilis*, which metabolized some carbohydrate and produced several new peaks. Each *Bacteroides* species produced typical HPLC patterns that could be used for taxonomic purposes. Table I shows utilization index data for the *Bacteroides* species examined. In addition to the carbohydrate peaks, eight significant new peaks (A–H) were produced. The production index data for these new peaks are shown in Table II. Peak A (retention time 14 min) was eluted between glucose and lyxose, peaks B, C and D (retention times 16, 19 and 23 min, respectively) were eluted between arabinose and salicin and peaks E, F, G and H (retention times 26, 30, 34 and 41 min, respectively) were eluted after salicin.

The *Bacteroides* species examined could be differentiated by their carbohydrate utilization patterns and the formation of new peaks. *B. vulgatus* was the only *Bacteroides* species that produced peak A whereas the production of peak H was a metabolic feature of *B. fragilis*. *B. ovatus* could be distinguished from the other species by the extent of its arabinose utilization. The remaining two species differed mainly with respect to lactose utilization, as the isolates of *B. thetaiotaomicron* strongly utilized lactose (utilization index 0.08) and those of *B. distasonis* only to a small extent.

TABLE II

NEW PEAKS PRODUCED BY *BACTEROIDES* SPECIES

Species	*Production index**							
	A	*B*	*C*	*D*	*E*	*F*	*G*	*H*
B. fragilis	0	1.07	2.83	0.77	0.55	2.55	1.33	1.99
B. thetaiotaomicron	0	0.69	2.25	0.73	2.08	2.77	0.67	0
B. ovatus	0	0.07	0.24	0.10	0.07	0.45	0.17	0
B. vulgatus	2.02	0.43	0.07	0.07	0.25	0.35	0.41	0
B. distasonis	0	0	0.03	0.01	0	0.07	0.01	0

* Data represent the mean values of the results obtained for the individual *Bacteroides* strains tested.

DISCUSSION

We have evaluated the HPLC monitoring of a chemically defined medium for the rapid identification of *Bacteroides* species. The medium was formulated on the basis of carbohydrate utilization results for *Bacteroides* species reported by other investigators[7]. Preformed enzymes appear to be responsible for carbohydrate utilization as all incubations were performed aerobically.

Utilization of the medium by *Bacteroides* resulted in the production of eight new peaks. Peak A, which was eluted with mannose, had the same retention time as pyruvic acid. Elimination of raffinose from the medium caused the size of peak A, produced by *B. vulgatus,* to decrease appreciably, suggesting that the formation of this peak could result from fermentation of raffinose. Peaks B, C and D have the same retention times as succinic, lactic and acetic acid, respectively. These acids are known to be metabolic products of *Bacteroides* species. Peaks F and G have retention times very close to those of isobutyric and butyric acid, respectively. However, peak F might correspond to a mixture of chemical entities, as the ultraviolet spectrum of the isolated component differed from that of a carboxylic acid.

This HPLC procedure offers the considerable advantage of yielding information about both the carbohydrate utilization and the metabolic end-products, simultaneously. To obtain similar information, it would be necessary to carry out both the biochemical tests of the API 20 A system and the gas chromatographic determination of fatty acids. Moreover, our procedure appears to be much more reliable than biochemical tests, mainly because the carbohydrate utilization by *Bacteroides* species can be determined quantitatively.

The possibility of increasing specificity and sensitivity by applying pre- or post-column derivatization reactions would enhance the utility of this method and provide a more precise identification of the metabolic products of a larger number of *Bacteroides* species[8,9].

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