Rapid Differentiation of Certain Bacteria in Mixed Populations by Gas–Liquid Chromatography[1]

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Although cellular alterations occurring during infection may reflect a general response to perturbation associated with pathogenesis rather than a specific response to the virulent agent (1, 2), the biochemical changes effected in the host by a pathogen may be highly specific and detectable by ultrasensitive gas–liquid chromatographic (glc) techniques (3, 4). However, such chromatographic analyses used in identification of bacteria responsible for infections may be extremely difficult or impossible to interpret if several species are concomitantly involved in the infection. The present preliminary report addresses this problem using gas chromatographic methods for defining organisms present in a selected mixed bacterial population *in vitro* and *in vivo*.

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MATERIALS AND METHODS

Bacteria. The following organisms were used in these studies: *Bacillus subtilis* (ATCC No. 6051), *Streptococcus faecalis* (α-haemolytic, group D, ATCC No. 19433), *S. pyogenes* (β-haemolytic, group A, ATCC No. 9342), *Escherichia coli* (ATCC No. 23783), *Alcaligenes* sp. (ATCC No. 10153), *Staphylococcus aureus* (ATCC No. 19636), *Salmonella typhimurium* (ATCC No. 13311) and *Pseudomonas aeruginosa* (ATCC No. 10197).

In vitro *cultures.* Bacteria were maintained on Trypticase soy agar and test inocula prepared from 16- to 24-hr cultures grown in Trypticase soy broth (TSB) at 37°C. The cells were removed from the liquid by centrifugation. Bacteria were enumerated by surface plating rabbit blood agar plates with 0.1-ml samples of serial dilutions in normal saline solution. An 0.1-ml inoculum containing 10^6 cells was inoculated into 8.0 ml of TSB and incubated for 24 hr at 37°C. The spent media were used for glc analysis.

Mixed cultures were prepared by inoculating two or three species of separately grown bacteria into tubes of the same medium, and the bacteria in these mixtures incubated 24 hr at 37°C. The uninoculated medium and the supernatant fluids of pure and mixed cultures were stored at −20°C for 1–2 wk until they were analyzed by gas–liquid chromatography (glc).

In vivo *studies.* Male, CD, rats (Charles River Breeding Laboratories, Willmington, Mass.), weighing 150–200 g were used for *in vivo* studies. The experimentally induced pure or mixed bacterial infections were studied by dividing healthy rats into four groups, each of which contained 20 animals. The first group was infected with 1 ml of sterile physiological saline solution containing 10^6 *Salmonella typhimurium* cells, the second group was inoculated with 10^6 *Pseudomonas aeruginosa* cells, the third was inoculated with a mixture of *S. typhimurium* (10^6 cells) and *P. aeruginosa* (10^6 cells), and the fourth received 1 ml of sterile saline solution. All the animals were inoculated intraperitoneally.

Gas–liquid chromatographic techniques. Glc of serum samples, the culture supernatant fluids, and the uninoculated media was performed by the previously reported methods (5, 6) with the following modifications: Immediately before extraction with the pyridine solvent a 2-ml sample was thawed and incubated with 2 ml of 8 N methanolic HCl for 2 hr at 70°C. By this procedure the protein components of the sample were precipitated and the pH was adjusted to about 2.0. The sample was then centrifuged, and the supernatant liquid was neutralized with NH_4OH and lyophilized. An equal amount of hydroxylamine was added to the dried material in order to stabilize the keto group of compounds by converting them into their oxime derivatives. The same mixture was then dissolved in 0.5 ml of pyridine by heating in a water bath at 75°C. The solution was then cooled to room temperature and silyl derivatives were formed by adding 0.2 ml hexamethyl disilazane (HMDS) and 0.1 ml trimethyl chlorosilane (TMCS). The mixture was shaken for 30 min and then centrifuged. A 3-μl aliquot of the clear supernatant solution was introduced into a gas chromatograph, model 207 (Varian Aerograph Co., Walnut Creek, Calif.) which was equipped with an electron capture detector (ECD) and a flame ionization detector (FID). The ECD (H^3-source) was operated at the maximum sensitivity range of 10^{-10} A/mV for the electronegative compound in the samples. The FID was used as a reference detector for internal standards and it was operated at 10^{-11} A/mV sensitivity range. The stainless-steel chromatographic col-

umn was 6 ft long and had a $\frac{1}{8}$-inch outer diameter, and it was packed with 10% Carbowax 20M coated on Chromosorb W (AW, HMDS treated) (Varian Aerograph Co., Walnut Creek, Calif.). The operating temperatures were 110, 190, and 210°C for the column, detector and injector, respectively. Other glc conditions were similar to those previously described.

RESULTS

Growth of bacteria in pure and mixed cultures. The number of organisms in pure cultures were 4×10^8 to 4×10^9 cells/ml after 24 hr growth in TSB media.

Lower numbers of viable cells were found when the two or three bacterial species were grown in the same culture medium tube (Table 1). The growth of *Streptococcus* species was inhibited by 100-fold when the organisms were inoculated with *B. subtilis* into TSB medium. The other organisms used in this study showed a 10-fold inhibition of growth in mixed cultures as compared to their growth in pure cultures (Table 1). However, both organisms grew well in *in vitro* cultures as indicated by the large cell numbers in samples taken at the time of glc analysis (24 hr postinoculation).

The growth patterns of *S. typhimurium* and *P. aeruginosa* in pure and mixed cultures were followed at various time intervals. As shown in Table 2, the pure cultures of *S. typhimurium* showed a rapid, progressive multiplication of the organisms *in vitro* as well as *in vivo*. *Pseudomonas aeruginosa* organisms showed similar growth pattern in *in vitro* cultures. However, the growth of the organisms in experimental rats reached a peak concentration of 10^7 cells/ml blood sample at 12 hr postinoculation and then declined (Table 2).

The organisms in a mixed population of *S. typhimurium* and *P. aeruginosa* grew well as indicated by their cell counts in samples taken at various intervals postinoculation (Fig. 1). The inoculum for these experiments contained 10^6 cells of

TABLE 1

GROWTH OF BACTERIA IN TRYPTICASE SOY BROTH INOCULATED WITH A
SINGLE SPECIES OR MIXED SPECIES OF ORGANISMS[a]

Pure cultures	Number of cells/ml culture[b] (24 hr after inoculation)
Bacillus subtilis	5×10^8
Staphylococcus aureus	6×10^8
Streptococcus pyogenes	4×10^8
Streptococcus faecalis	3×10^9
Escherichia coli	4×10^9
Alcaligenes sp.	8×10^8

Mixed cultures	
B. subtilis + *S. aureus*	1×10^7 (*B. subtilis*); 8×10^7 (*S. aureus*)
B. subtilis + *S. pyogenes*	4×10^7 (*B. subtilis*); 5×10^6 (*S. pyogenes*)
B. subtilis + *S. faecalis*	3×10^7 (*B. subtilis*); 6×10^7 (*S. faecalis*)
B. subtilis + *E. coli*	1×10^7 (*B. subtilis*); 5×10^8 (*E. coli*)
B. subtilis + *Alcaligenes sp.*	4×10^7 (*B. subtilis*); 4×10^7 (*Alcaligenes sp.*)
B. subtilis + *Alcaligenes sp.* + *E. coli*	6×10^7 (*B. subtilis*); 1×10^7 (*Alcaligenes sp.*) 6×10^7 (*E. coli*)

[a] Bacteria were isolated on blood agar plates and identified by colonial morphology.
[b] The values represent an average of six determinations.

TABLE 2

GROWTH OF *Salmonella Typhimurium* AND *Pseudomonas Aeruginosa*
ORGANISMS IN PURE CULTURES INOCULATED INTO TRYPTICASE
SOY BROTH AND EXPERIMENTAL ANIMALS[a]

Incubation	S. typhimurium		P. aeruginosa	
time (hr)	In vitro[b]	In vivo[c]	In vitro[b]	In vivo[c]
0	0	0	0	0
6	5×10^4	1×10^4	1×10^4	—
12	2×10^6	8×10^4	2×10^4	1×10^7
24[d]	6×10^8	2×10^6	5×10^7	4×10^6
48	3×10^9	4×10^8	4×10^8	8×10^5
72	4×10^9	6×10^8	9×10^8	2×10^4

[a] The values represent an average of four determinations.
[b] Bacteria/ml culture media.
[c] Bacteria/ml blood.
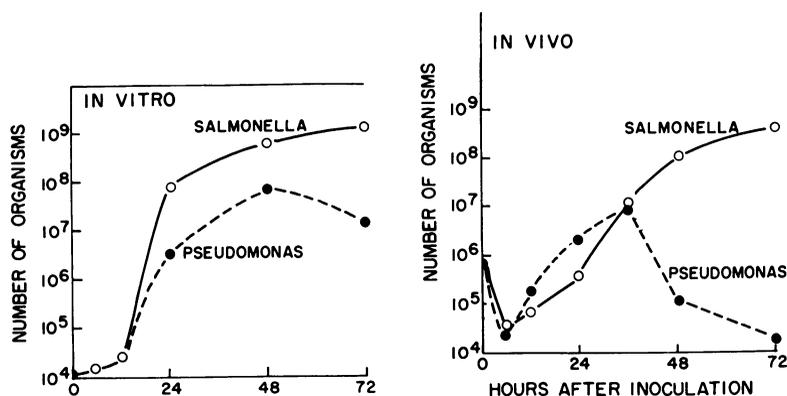[d] Samples were used for gas–liquid chromatographic analysis in this study.

FIG. 1. Growth of *Salmonella typhimurium* and *Pseudomonas aeruginosa* in mixtures in *in vitro* cultures and in experimental animals. Each point on the graph represents an average value of four determinations. Samples taken at 24 hr after inoculations were used for gas–liquid chromatography analysis in this study.

each species grown separately and mixed prior to inoculation. The number of *P. aeruginosa* in the mixed *in vitro* cultures was maximum (10^8 cells/ml) at 48 hr but declined at 72 hr postinoculation. The growth of *P. aeruginosa in vivo* declined rather rapidly 36 hr postinoculation of the two bacterial species. *Salmonella typhimurium* organisms had larger concentrations of bacterial cells *in vitro* and *in vivo* in the mixed infections with *P. aeruginosa* (Fig. 1). Acute bacteremia in rats resulted at 72-hr postinoculations of *S. typhimurium* either in pure cultures or mixed with *P. aeruginosa* cells. No samples were taken for bacterial counts after 72-hr inoculations in this study.

Glc analysis of in vitro *cultures. Bacillus subtilis* organisms were used in this study to prepare mixed cultures with other known species of bacteria. The chromatographic analysis of *B. subtilis* pure culture revealed that the peaks with retention times (Rt) of 160 (±2), 200 (±2), 260 (±3), 325 (±3), 350 (±4), 490 (±4), 560 (±6), 805 (±8), 1310 (±14), 2010 (±21) and 3595 (±37) sec-
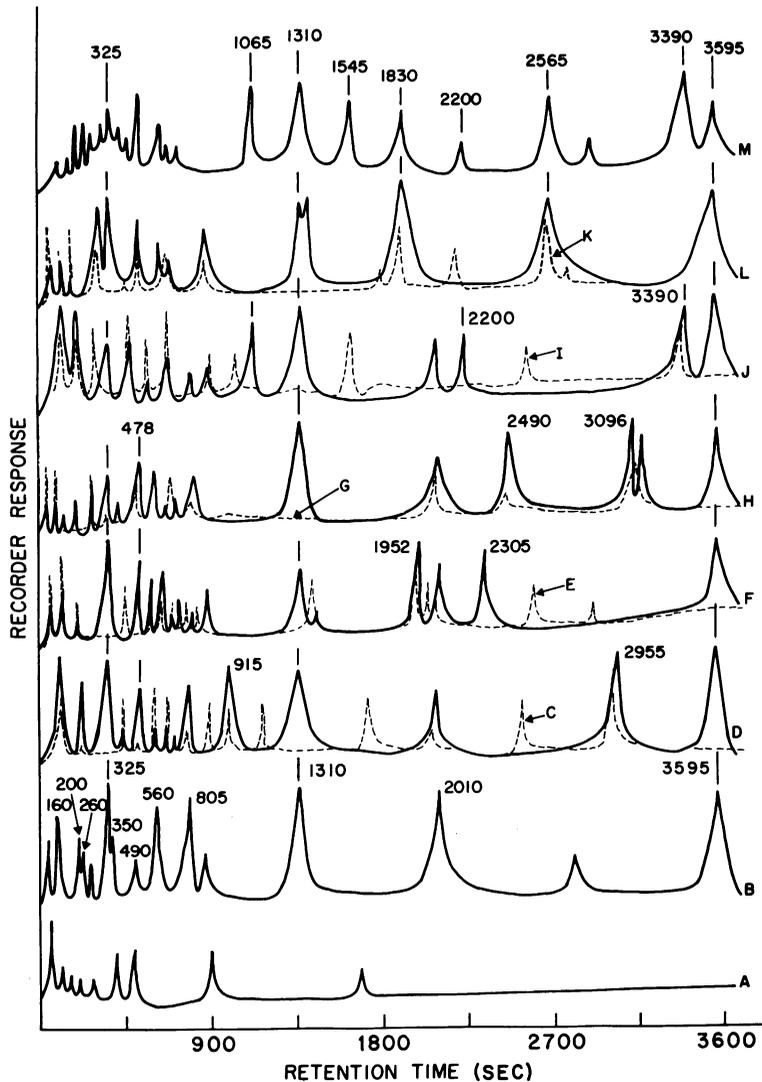
FIG. 2. Gas chromatographic analysis of products elaborated by bacteria growing in mixed populations. The broken lines represent the chromatographic patterns of pure cultures of bacteria in their respective mixed cultures (solid lines). The labels on the peaks designate their retention times, in seconds, as sensed by the electron capture detector. (A) uninoculated medium; (B) *Bacillus subtilis;* (C) *Staphylococcus aureus;* (D) *S. aureus* and *B. subtilis;* (E) *Streptococcus pyogenes;* (F) *S. pyogenes* and *B. subtilis;* (G) *S. faecalis;* (H) *S. faecalis* and *B. subtilis;* (1) *Escherichia coli;* (J) *E. coli* and *B. subtilis;* (K) *Alcaligenes* species; (L) *Alcaligenes* sp. and *B. subtilis;* and (M) *Alcaligenes* sp., *B. subtilis* and *E. coli.*

onds were consistently present in all the replicate cultures examined (Fig. 2). Some of these compounds were also found in the uninoculated culture medium. However, the compounds (peaks) with Rt values of 325, 560, 805, 1310, 2010, and 3595 sec were not found in the uninoculated culture medium. From our previous studies, it was observed that several compounds with the same Rt values (i.e., 325, 805, 1310, 2010 and 3595 seconds) were also detected in the serum samples of rats inoculated intraperitoneally with *B. subtilis* (unpublished data).

Metabolites characteristic of this *B. subtilis* culture were likewise noted when it was grown in mixtures with *S. aureus, S. pyogenes, S. faecalis, E. coli* and *Alcaligenes* sp. (Fig. 2). In these mixtures, peaks with Rt of 325, 1310 and 3595 seconds specifically indicated the presence of *B. subtilis* since these peaks were always found in the chromatograms of the mixed cultures, and they were absent in cultures lacking the *B. subtilis* organism. The other bacterial species growing in a mixed population with *B. subtilis* also showed certain characteristics peaks. They had Rt values of 915 (±10) and 2955 (±30) seconds for *S. aureus;* 1952 (±19) and 2305 (±22) seconds for *S. pyogenes;* 478 (±5), 2490 (±25) and 3096 (±32) seconds for *S. faecalis;* 1065 (±12), 2200 (±20), 3390 (±32) seconds for *E. coli;* and 1830 (±17) and 2565 seconds peaks for *Alcaligenes* sp. Chromatograms of pure cultures, shown as broken lines in Fig. 2, had many characteristic peaks similar to those found in their mixed cultures. However, there were also some differences in the chromatographic patterns of pure cultures of the organisms as compared to their respective mixed cultures (Fig. 2). The identities of these peaks were not determined. However, from the extraction procedures and glc conditions used, it was estimated that these characteristic metabolites were bi- and tricarboxylic acids and polyhydroxy aldehydes or ketones.

Glc analysis of serum samples. The results of glc analysis of the metabolites present in serum samples obtained from rats inoculated with pure or mixed cultures of *S. typhimurium* and *P. aeruginosa* are presented in Table 3. Serum samples taken 24 hr after inoculations were used for this study. It is evident that compounds of Rt values of 180 and 240 sec which were present in mixed infections were absent from the blood of animals inoculated with a single bacterium. However, several of the metabolites in the mixed infection were chromatographically identical to products generated in rats receiving a single microbial species (e.g., compounds

TABLE 3

RETENTION TIMES OF CHARACTERISTIC METABOLITES IN SERA OF RATS
INFECTED WITH PURE AND MIXED BACTERIAL CULTURES[a]

P. aeruginosa infection		*S. typhimurium* infection		Mixed *S. typhimurium, P. aeruginosa* infection	
Retention time (sec)[b]	Peak area (mm²)[c]	Retention time (sec)[b]	Peak area (mm²)[c]	Retention time (sec)[b]	Peak area (mm²)[c]
510 (±5)[d]	2200–2600	*395 (±4)[d]*	1200–1600	*180 (±2)[d]*	1100–1400
780 (±8)[d]	800–1000	*450 (±5)[d]*	2000–2400	*240 (±2)[d]*	500–800
870 (±8)[d]	900–1400	*590 (±5)[d]*	1000–1400	*510 (±5)[d]*	1000–1400
1050 (±10)	2600–3200	**700 (±8)**	2600–2900	**700 (±8)**	600–900
1140 (±11)	2000–2400	**870 (±9)**	1300–1700	**870 (±9)**	1400–1800
1350 (±14)[d]	1000–1400	**1200 (±10)[d]**	1800–2200	*1050 (±11)[d]*	1200–1600
1800 (±16)	1800–2200	**1230 (±12)[d]**	2700–3000	*1140 (±12)*	2100–2500
2100 (±20)[d]	2800–3000	**1650 (±17)**	2900–3000	*1200 (±10)[d]*	2000–2300
		2070 (±20)[d]	2800–3200	*1350 (±14)[d]*	1800–2200
		2400 (±25)	1800–2100	**1650 (±16)**	3000–3400

[a] The compounds were not found in sera of uninoculated, control animals.

[b] Retention times are based on average determinations of sera from 20 rats. Standard deviations are given in parenthesis. Italicized retention times represent common peaks due to *P. aeruginosa* activity and the boldface due to *S. typhimurium*.

[c] The range of peak areas.

[d] Compounds with the same retention times were also found in 24-hr cultures grown in trypticase soy broth.

of Rt 510, 1050, 1140 and 1350 sec were associated only with *P. aeruginosa* infections). Several of these compounds found in sera of pure or mixed cultures inoculated animals were also present in their *in vitro* cultures (Table 3). Sham-inoculated rats revealed none of these substances. Bacteremia resulted from these mixed infections, and both species of bacteria were isolated from heart blood sample taken 72 hr postinfection.

DISCUSSION

The objective of this study was to determine whether gas–liquid chromatographic methods can be used to identify the presence of individual strains in a bacterial mixture. The data show that bacteria generate characteristic metabolites in pure as well as mixed cultures, and these compounds can be detected from their spent culture media and sera by glc means. In the *in vitro* mixed cultures, at least two metabolites associated with the metabolic activity of individual bacterial species were sensed by these glc procedures. It is essential, however, that all the species of organisms in a mixed population be viable in order to produce certain identifiable products by glc techniques. The results of this study show that the growth of each species of bacteria in the mixed cultures were about 10^7 cells/ml as compared to their pure cultures which were in the range of 10^8–10^9 cells/ml. This reduction in proliferation of organisms in mixed cultures may be due to competition in nutritional substrates in the media. However, the growth patterns of each species in these defined mixtures indicate that there was no direct interference or inhibition of one species of bacteria by the other species. It was interesting to note that several products of an individual species of bacteria in the mixed cultures were chromatographically identical to those found in pure culture (Fig. 2). However, there were some different peaks present only in pure cultures and other peaks found in mixed cultures. For example, the peaks with Rt values of 805, 1140, 1750 and 2520 sec were noted in *S. aureus* pure cultures (Fig. 2C) whereas peaks with Rt values of 1310 and 3595 were present in the cultures of *S. aureus* and *B. subtilis* mixed populations (Fig. 2D). Similar chromatographic patterns were observed with the analysis of pure and mixed cultures of other bacterial species. These differences in the products of organisms in mixed cultures may be related to the influence exerted by different metabolic activities of the organisms. Nevertheless, at least two metabolites associated with the metabolic activity of individual bacterial species were sensed by these chromatographic procedures. These results are in agreement with our previous findings with viruses (5) and pure cultures of bacteria (6).

Little is known about the metabolism of pathogenic bacteria developing within their animal hosts. Infective bacteria of two or more different kinds are known to coexist, each bacteria type expressing its own biochemical potential in the host body environment (7, 8) but one or another usually predominates depending upon prevailing conditions. The results of this study support this point of view. When *S. typhimurium* and *P. aeruginosa* were inoculated into rats to induce mixed infections, the growth of *S. typhimurium* predominated. The experimental animals were able to overcome *P. aeruginosa* infections (pure or mixed with *S. typhimurium*) and after 72-hr inoculations a low number (10^4 cells/ml) of *P. aeruginosa* organisms were found. However, the blood samples taken from infected animals were at 24 hr postinoculation when both organisms were in a logarithmic growth phase indicated by the high cell numbers (10^6 cells/ml each of *S. typhimurium* and *P. aeruginosa* organisms) (Table 2).

It was interesting to note that several of the peaks observed for infections with a single organism are not found in mixed infections (780, 1800 and 2100 sec for *P. aeruginosa;* 395, 450, 590, 1230, 2070 and 2400 sec for *S. typhimurium*). Also, new peaks are found in mixed infections (180 and 240 sec) (Table 3). These peaks may be associated with the nonspecific activity of individual species of organisms in pure or mixed cultures. However, there were certain peaks which may be related to the specific activity of *P. aeruginosa* (510, 870, 1050, 1140 and 1350 sec) and *S. typhimurium* (700, 870, 1200 and 1650 sec) since these peaks were also found in their mixed cultures. Also, several of these compounds with the same retention times were detected in 24-hr cultures grown in TSB (e.g., 510, 870 and 1350 sec for *P. aeruginosa* and 1200-sec peaks for *S. typhimurium.*) The mechanism of production of these compounds *in vitro* and *in vivo* is not known at this time. Since there are several factors such as interactions of the organisms in a mixed population, host response and the differences in metabolic activity of organisms *in vitro* and *in vivo*, it is difficult to explain the chromatographic patterns of mixed infections. Nevertheless, by using the criteria of reproducibility and accuracy of certain sets of peaks associated with the activity of an individual strain of bacteria, the glc method may resolve the identity of bacteria in a mixed population.

It would be highly desirable to determine the identities of the peaks associated with each organism. Gas–liquid chromatography in conjunction with mass spectrometry has been recently described for identification and structural studies of compounds in multicomponent biological materials (9, 10). These methods will be very useful in the identification of the characteristic compounds associated with individual bacterial species in a mixed infection. However, by using selective extraction and hydrolysis procedures, and method of derivative formation together with the use of known standard chemicals, bacterial products could be characterized in certain groups of compounds. In this study, although the peaks were not identified, the glc procedures were selective for analyzing carboxylic acids and polyhydroxy aldehydes and ketones in the culture media and sera. In a previous study selective gas chromatographic analysis of serum samples from *D. pneumoniae* infected rats for mucopolysaccharides and glycoprotein components showed characteristic metabolic changes at the early stages of infection (11). Products characteristic of individual groups of bacteria associated with human infections have also been noted; thus differentiation of seven types of bacterial infections in man was facilitated by similar gas chromatographic procedures (12). These results indicate that similar methods have potential applications in rapid identification of bacteria in mixed infections.

SUMMARY

Gas–liquid chromatographic techniques were employed to differentiate bacteria growing in defined mixtures. Retention times characteristic of individual bacterial populations were observed in mixed cultures containing two or three bacterial species growing *in vitro*. Gas chromatograms of serum samples from rats infected with two different species of *S. typhimurium* and *P. aeruginosa* showed chromatographically distinguishable metabolites associated with the activities of the two organisms. It is suggested that bacteria growing in mixtures may be identified *in vitro* as well as *in vivo* by these chromatographic techniques.

ACKNOWLEDGMENTS

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