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# CAPILLARY GAS CHROMATOGRAPHY–MASS SPECTROMETRY OF CARBOHYDRATE COMPONENTS OF LEGIONELLAE AND OTHER BACTERIA

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## SUMMARY

Capillary gas chromatography using fused-silica columns followed by electron impact or chemical ionization mass spectrometry was used to profile and identify neutral and amino sugars present in several legionellae and other bacteria. A modified alditol acetate derivatization method was employed to produce volatile carbohydrate derivatives. Muramic acid, a component of bacterial peptidoglycan, was detected in all legionellae examined. Heptose, a component of bacterial lipopolysaccharide, was identified in *Escherichia coli* organisms and in several purified Gram-negative bacterial lipopolysaccharides but not in the legionellae examined. Two amino dideoxyhexoses were found to be present in several of the Legionellae examined. The potential of gas chromatography–mass spectrometry for the direct chemical characterization of microorganisms is discussed.

## INTRODUCTION

Capillary gas chromatography (GC) is a powerful technique for the analysis of complex biochemical samples such as bacterial cells or components derived from microorganisms. The direct characterization of microorganisms by GC offers several advantages over traditional techniques based on serological, morphological, or biochemical tests, particularly in regard to speed of analysis, specificity, and sensitivity. The use of GC to separate and identify chemical constituents of microorganisms is particularly useful where specific chemicals exist that are characteristic of certain groups of microorganisms. For example, substantial amounts of the amino sugar muramic acid are found in the cell wall of most bacteria and, because muramic acid is not found elsewhere in nature, its detection in a sample implies the presence of bacteria or bacterial fragments[1,2]. Another chemical marker, heptose, is generally

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found in the lipopolysaccharide of Gram-negative bacteria[3]. In many other cases, components might be present that are specific to certain classes of microorganisms. The relative amounts of common components such as rhamnose, fucose, or glucosamine might also permit differentiation or provide information concerning the fundamental chemical structure of the microorganism.

GC with non-selective detection (such as a flame ionization) does not permit unequivocal identification of chromatographic peaks. Identification of components based only on retention time is especially difficult in the analysis of bacterial samples where the number of possible constituents is quite large and where closely related microorganisms can have unique constituents with similar retention times. Pretreatment steps to clean up the sample prior to GC are essential in simplifying the resulting chromatogram and making its interpretation easier. The coupling of capillary GC to mass spectrometry (MS) offers further advantages including improved selectivity and the potential for completely identifying or at least partially characterizing the chemical nature of the constituents of interest.

The analysis of chemical constituents in microorganisms by GC or GC–MS has been reviewed by Drucker[4] and Moss[5]. Recently, Larsson *et al.*[6] employed GC–MS with selected ion monitoring (SIM) to detect tuberculostearic acid in mycobacteria and nocardia. Corina and Sesardic[7] have also used GC–MS to profile and identify mycolic acids from corynebacteria. Moss and coworkers[8–10] used GC and GC–MS to determine the branched chain fatty acid composition of *Legionellaceae*. Brice *et al.*[11] employed GC with flame ionization detection to profile carbohydrates from several bacterial species that cause meningitis. The carbohydrate composition of several strains of streptococci have been determined by Aluyi and Drucker[12] using GC–MS and by Pritchard *et al.*[13] using GC with an electron capture detector. Fatty acids and carbohydrates from several strains of mycobacteria have been analyzed by Alvin *et al.*[14].

Previous work in our laboratories has focused on the development of improved methods for the analysis of carbohydrates in bacterial samples using a modified alditol acetate derivatization procedure followed by glass capillary GC[15,16]. We have also recently reported the use of carbohydrate profiles of intact bacterial cells to differentiate members of the family *Legionellaceae*[17]. This article presents the application of fused silica capillary columns coupled to mass spectrometry for the characterization and identification of a number of neutral and amino sugars in *Legionella* and other bacteria.

## EXPERIMENTAL

### *Bacterial strains, media and preparation of bacterial cells*

*Legionella pneumophila* philadelphia 1, *Tatlockia micdadei* (TATLOCK), *Fluoribacter bozemanae* (WIGA), and *Fluoribacter dumoffii* (NY 27) were incubated for three days on buffered charcoal yeast extract agar in air at 37°C. *Escherichia coli* (ATCC 25922) was grown in trypticase soy broth. *Streptococcus pyogenes* was grown in Todd-Hewitt broth. All of these organisms were washed in distilled water, then heat killed, and lyophilized. *Pasteurella multocida* lipopolysaccharide (LPS) was a gift from Dr. Paul Rebers (National Animal Disease Center, Ames, IA, U.S.A.) and *Salmonella typhimurium* LPS was purchased from Difco Laboratories (Detroit, MI, U.S.A.).

*Chemicals*

L-rhamnose, L-fucose, D-xylose, D-mannose, D-galactose, and D-glucose standards were obtained from Supelco (Bellefonte, PA, U.S.A.). 2-Deoxy-D-ribose, D-ribose, muramic acid, D-glucosamine hydrochloride, D-galactosamine hydrochloride, and methylglucamine were obtained from Sigma (St. Louis, MO, U.S.A.). D-Glycero-L-mannoheptose was a gift from Dr. Paul Rebers.

Glass-distilled acetic anhydride (Applied Science Labs, State College, PA, U.S.A.), chloroform (Burdick & Jackson Labs., Muskegon, MI, U.S.A.) and methanol (Burdick & Jackson Labs.) were purchased. Reagent grade glacial acetic acid (J. T. Baker, Phillipsburg, NJ, U.S.A.) was glass-distilled prior to use. N,N-dioctylmethylamine was purchased from ICN (Plainview, NY, U.S.A.). Reagent grade sulfuric acid, sodium borohydride, and sodium borodeuteride were obtained from Fisher Scientific (Fairlawn, NJ, U.S.A.). Bond Elut hydrophobic extraction columns and Clin Elut hydrophilic columns were purchased from Analytichem International (Harbor City, CA, U.S.A.). All glassware was washed with detergent, distilled water, 1 *N* hydrochloric acid, and chloroform.

*Preparation of carbohydrate derivatives*

The procedures employed here for the hydrolysis of bacterial samples and for the derivatization of carbohydrate components were based on a previously published method[15]. Typically, 1 mg of lyophilized sample in 0.5 ml 2 *N* sulfuric acid was placed in a hydrolysis tube (Pierce, Rockford, IL, U.S.A.). Ten tubes were connected simultaneously to a vacuum manifold that was connected to both a nitrogen tank and a vacuum pump. This manifold configuration permitted each hydrolysis tube to be alternately evacuated and flushed with nitrogen to remove oxygen. The PTFE valves on the hydrolysis tubes were closed under vacuum and hydrolysis was performed in a Pierce (Rockford, IL, U.S.A.) Reacti-Therm heating module at 100°C for 3 h. After the tubes were cooled to room temperature, 50 μl of internal standard (arabinose for neutral sugars and methylglucamine for amino sugars) and 2.5 ml of 20% N,N-dioctylmethylamine in chloroform were added. The mixture was vigorously mixed on a Vortex mixer and allowed to settle into two layers on standing. A 1-ml Bond Elut column was prepared for each sample by rinsing with 2 ml methanol followed by 2 ml of distilled water. The upper aqueous layer of the hydrolysis mixture was carefully pipetted onto the column along with 50 μl of freshly prepared sodium borohydride solution (100 mg/ml). Each mixture was then pulled through the column by vacuum into a reaction vial fitted with a screw cap and a PTFE silicone liner (Pierce) and the column was washed with 1 ml of distilled water. The Vac-Elut system (Analytichem) permitted up to 10 samples to be run simultaneously. The reduction was allowed to proceed overnight in a refrigerator. Excess sodium borohydride was destroyed by adding 2 ml of acetic acid–methanol (1:200, v/v) to the sample which was then evaporated to dryness in a Vortex Evaporator (Buchler, Ft. Lee, NJ, U.S.A.) at 60°C under vacuum. This step was repeated four additional times to insure complete removal of the sodium borohydride. The vials were allowed to dry for 3 h after the last evaporation. After cooling to room temperature, 300 μl of acetic anhydride was added to each vial and the sample was heated to 100°C for 13–16 h on the Reacti-Therm heating module (Pierce). The samples were then evaporated to dryness. One ml of water and 1 ml of chloroform were added to each vial and the

mixture was vortexed prior to pouring onto a 1 ml Clin Elut column. The sample was eluted with 3 ml chloroform and evaporated to dryness. The alditol acetate samples were redissolved in a minimal volume of chloroform (typically 20–30 μl) and 1 μl aliquots were injected into the GC–MS with a microsyringe.

*Capillary gas chromatography–mass spectrometry*

A blank 0.2 mm × 25 m fused-silica capillary column was obtained from Hewlett-Packard (Avondale, PA, U.S.A.). The column was static coated with SE-52 (Supelco) in our laboratory and treated with dicumyl peroxide (Polysciences, Warrington, PA, U.S.A.) to crosslink the stationary phase in a manner similar to that described by Grob *et al.*[18]. The column was rinsed with solvents and conditioned prior to use. The injection port temperature was 250°C. The split vent flow-rate was 20 ml/min and the column was operated with a head pressure of 10 p.s.i. The column oven temperature program started at 150°C, was programmed at 4°C/min to 210°C, then at 10°C/min up to 260°C and held at that temperature until all components of interest eluted.

A Finnigan (Palo Alto, CA, U.S.A.) Model 4021C GC–MS instrument was used for all experiments. Because the differential pumping system could handle the low flow of an open tubular column, the flexible fused-silica capillary was connected directly to the mass spectrometer permitting the entire column effluent to enter the ion source region without splitting. The use of a fused silica column in this manner minimizes the potential degradation or adsorption of sample components on active sites in the instrument.

## RESULTS AND DISCUSSION

Previous experience[17] in carbohydrate profiling has demonstrated the ability of capillary GC to distinguish samples from three different groups of the Legionellaceae: *L. pneumophila, T. micdadei* and *Fluoribacter* species. Fig. 1 shows total ion abundance chromatograms of representative samples from these three groups. Chromatograms from two different *Fluoribacter* species are included in Fig. 1. Two internal standards, arabinose and methylglucamine, were added to each sample during the derivatization procedure and were employed to quantitate each sugar. The following sections discuss the identification and significance of the alditol acetate peaks shown in Fig. 1.

*Neutral sugars*

The relative amounts of the early eluting neutral sugar peaks (rhamnose, ribose and fucose) permit some discrimination among the four samples shown in Fig. 1. Fucose is absent from the *L. pneumophila* and *F. dumoffii* chromatograms. The amounts of rhamnose and fucose are largest in the *T. micdadei* samples. Although the SP-2330 capillary column used in previous work[15–17] can better resolve alditol acetates of these neutral sugars than can the SE-52 column used here, the early eluting peaks can be clearly resolved with the added dimensions supplied by mass spectrometry. Fig. 2C shows the unresolved total ion abundance trace for the group of four components (ribose, rhamnose. fucose and arabinose). Plotting only ion mass 200 as

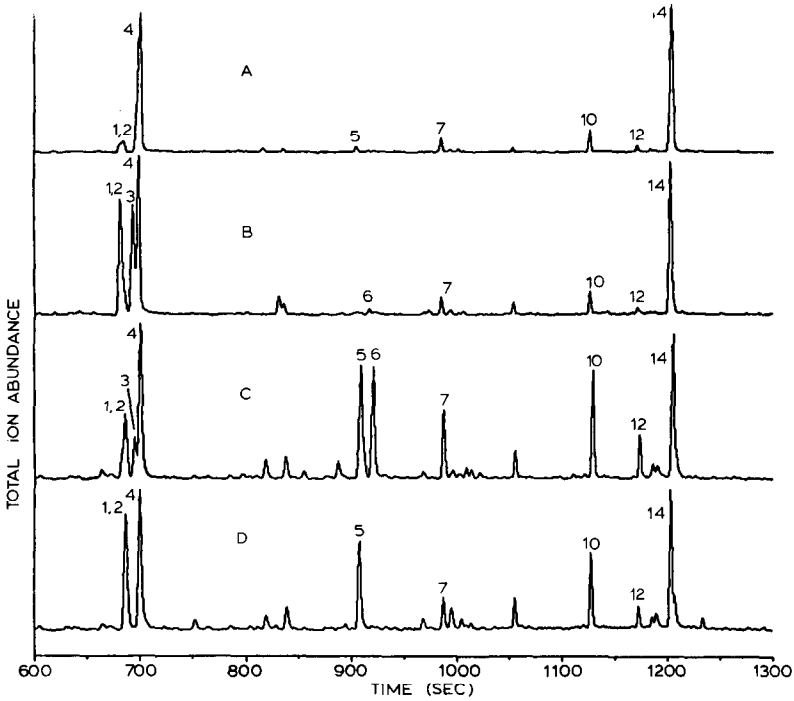



Fig. 1. Reconstructed total ion abundance chromatograms of the alditol acetates from (A) *L. pneumophila*, (B) *T. micdadei*, (C) *F. bozemanae* and (D) *F. dumoffii*. Peak identities are: 1 = rhamnose; 2 = ribose; 3 = fucose; 4 = arabinose (neutral sugar internal standard); 5 = amino dideoxy sugar X1; 6 = amino dideoxy sugar X2; 7 = mannose; 10 = glucosamine; 12 = muramic acid; 14 = methyl glucamine (amino sugar internal standard).

shown in Fig. 2A resolves the ribose and arabinose peaks, while plotting only ion mass 231 as shown in Fig. 2B resolves the rhamnose and fucose peaks.

Mannose was easily seen in all samples by both capillary GC and GC–MS; glucose and sometimes galactose, which elutes immediately after mannose, were detected in trace amounts (less than 1 $\mu$g/mg sample) by GC–MS but could not be detected with as high confidence by GC using FID detection[17]. Fig. 3 displays a total ion abundance chromatogram and a reconstructed ion chromatogram for mass 115 showing the region of the chromatogram where mannose, glucose, and galactose elute. Although the three peaks are significantly above the baseline in this case, the glucose and galactose peaks are quite small, making identification difficult with just the total ion abundance chromatogram. Plotting selected masses (in this case mass 115) aids in distinguishing these peaks from the background of other material eluting from the column at the same time. These three sugars, however, are present at high enough levels to permit identification by comparison of their complete mass spectra with a standard sample of the sugar carried through the derivatization procedure to produce the corresponding alditol acetate. Fig. 4 shows a comparison of the complete mass spectra of the alditol acetate of glucose from *L. pneumophila* with that of a glucitol hexacetate standard. Excellent correspondence between the two spectra is seen.

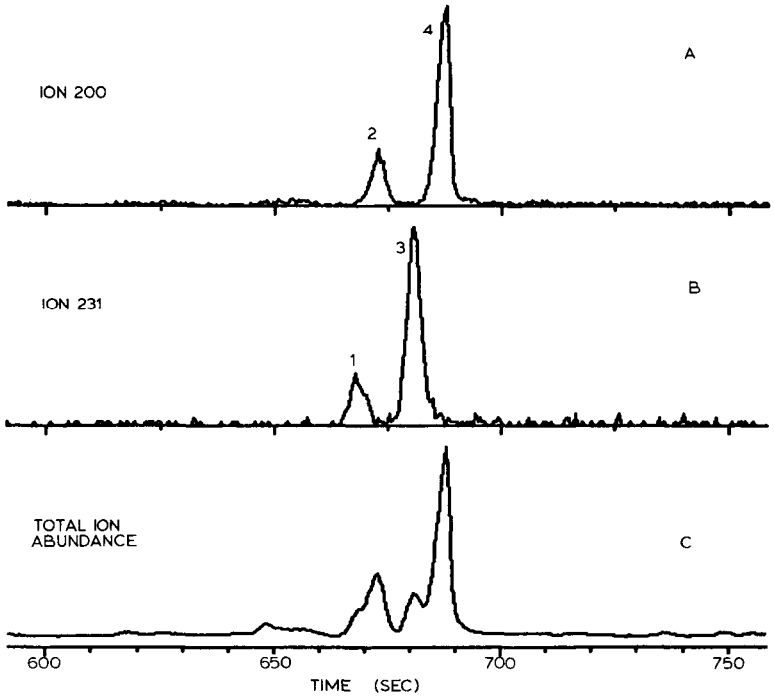



Fig. 2. Reconstructed ion chromatograms of the early eluting neutral sugar alditol acetates from *F. bozemanae*. Masses 200 and 231 are plotted above the total ion abundance (mass 40–450) chromatogram. Peak identities are: 1 = rhamnose; 2 = ribose; 3 = fucose; and 4 = arabinose (internal standard).

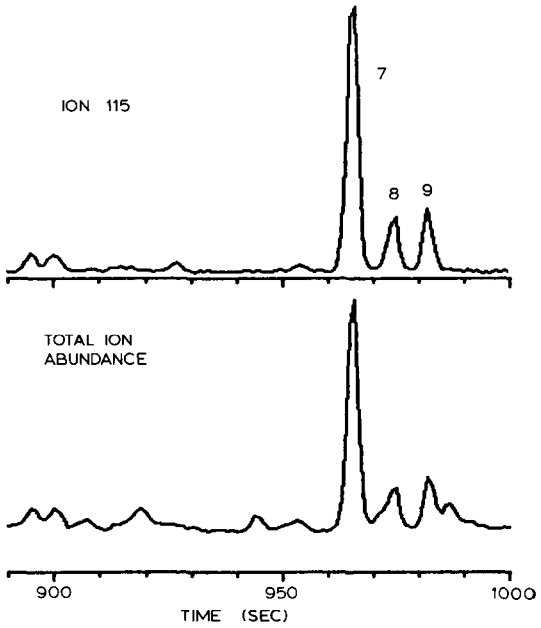



Fig. 3. Reconstructed ion (mass 115) and total ion (mass 40–450) plots of the portion of a chromatogram of alditol acetates derived from *L. pneumophila* showing the presence of mannose (7), glucose (8) and galactose (9).

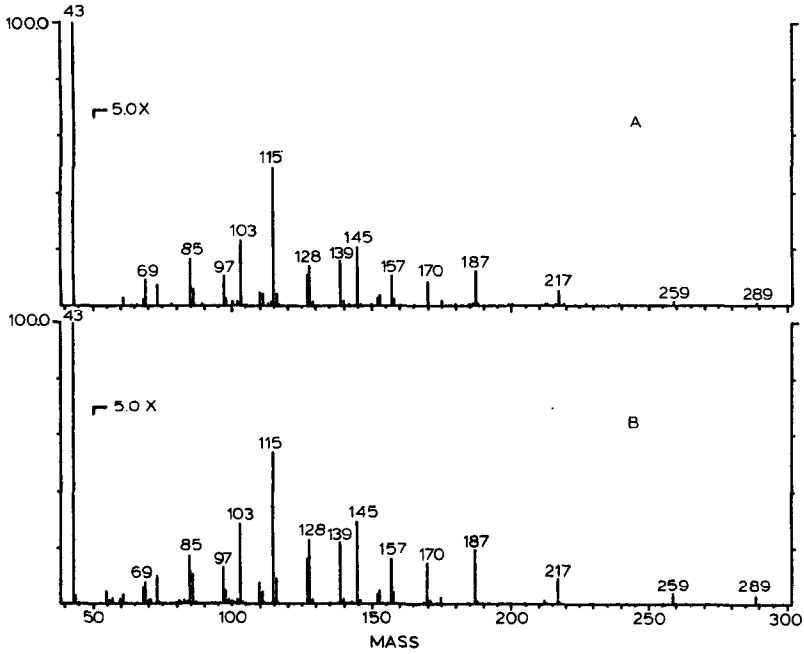



Fig. 4. Electron impact (70 eV) mass spectra of (A) the glucose alditol acetate derived from a sample of *L. pneumophila*, and (B) a glucose hexaacetate standard.

*Amino sugars*

One of the more important amino sugars found in bacteria is muramic acid, 3-O-(1′-D-carboxyethyl)-D-glucosamine. N-Acetylmuramic acid and N-acetylglucosamine are generally present in equal amounts in the carbohydrate backbone of the bacterial cell wall (peptidoglycan). This glycan polymer is a linear chain of alternating N-acetylmuramic acid and N-acetylglucosamine units crosslinked with peptide sidechains. Peptidoglycan comprises between 30 and 70% dry weight of the cell walls of Gram-positive organisms but only about 5 to 10% of the dry weight of Gram-negative cell walls[1]. We have identified muramic acid and glucosamine in every bacterial sample examined, however the amounts of glucosamine were always slightly higher than the amounts of muramic acid thus possibly indicating the presence of glucosamine elsewhere in the bacteria. Of the bacteria we have examined, galactosamine was found only in *E. coli* (see Fig. 9 below).

A methane chemical ionization (CI) mass spectra of the alditol acetate of muramic acid obtained from a sample of *F. bozemanae* is shown in Fig. 5. The retention time of this compound on the SE-52 capillary column and its mass spectra [both electron impact (EI) and CI] match the results obtained from a muramic acid standard. The CI spectra suggests that the alditol acetate of muramic acid has the cyclic structure superimposed on Fig. 5 rather than the free acid structure. The molecular weight of the cyclic structure is 445, whereas the molecular weight of the free acid form of muramicitol pentaacetate would be 463. The peak at mass 446 represents the (M + H) ion and the peak at mass 474 represents the addition of $C_2H_5$. The ions of mass 386 (M + H − 60) and 404 (M + H − 42) are formed by the loss of acetic

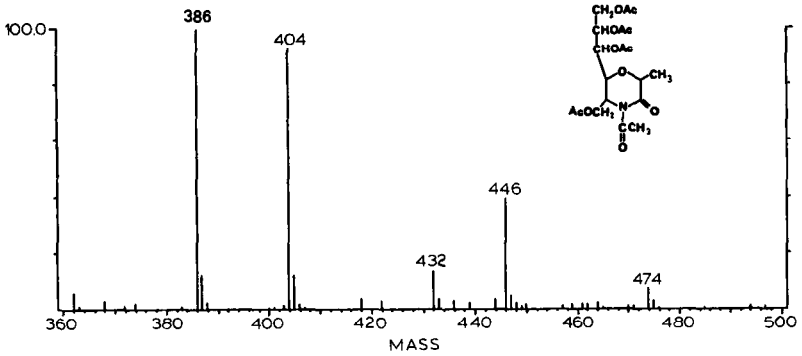



Fig. 5. Methane chemical ionization mass spectra of the alditol acetate of muramic acid from a sample of *F. bozemanae*.

acid and ketene respectively from the protonated molecular peak. The peak at mass 432 is due to a loss of ketene from the (M + $C_2H_5$) ion.

Two other rather unusual amino sugars were found in some of the legionellae examined. Peaks 5 and 6 (sugars X1 and X2), which occur prominently in the middle region of the chromatogram of *F. bozemanae* in Fig. 1 C, are together characteristic of that species. These two peaks do not occur in the same high amounts in the other chromatograms of Fig. 1. *F. dumoffii*, with a large amount of sugar X1 but no X2, is the only other sample with appreciable amounts of either component. The retention times of these two sugars did not match the retention times of any sugar standards available to us and their structural elucidation provided a major impetus for the use

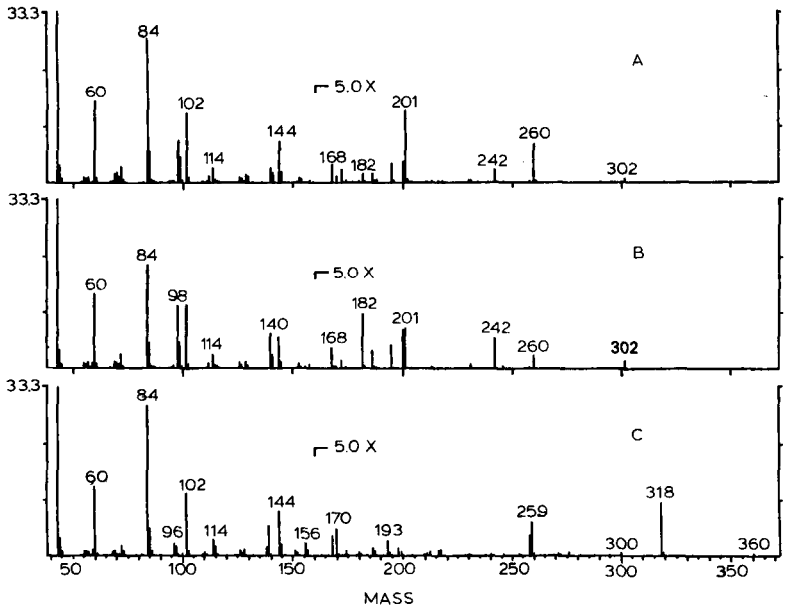
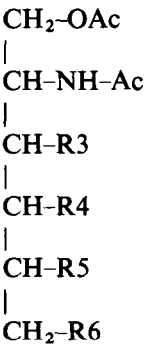


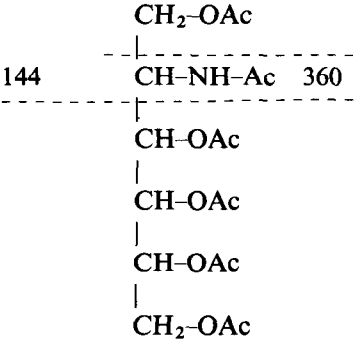

Fig. 6. Electron impact (70 eV) mass spectra of the alditol acetates of sugars (A) X1, (B) X2 and (C) glucosamine from a sample of *F. bozemanae*. The heights of peaks in the mass spectra are multiplied by 3 from mass 40–160 and by 15 from mass 160–370.

of mass spectrometry with these samples. We propose that the X1 and X2 sugars are isomers of 2-amino-2,*n*-dideoxyhexoses, where the *n* represents the position of the second deoxy carbon and could be either 3, 4, 5, or 6. The structure of the alditol acetate of this sugar can be represented as:



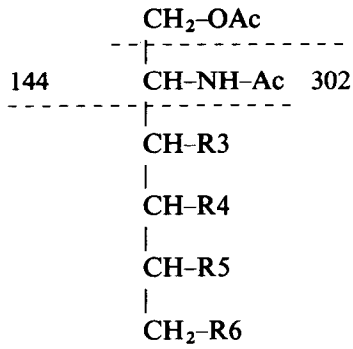

where one of the four R groups is a hydrogen (–H) and the other three are acetate groups (–O–CO–$CH_3$ or –OAc). The occurrence of other amino deoxy sugars, including some amino dideoxy sugars, in bacteria and other natural products has been reviewed by Horton and Wander[19].

Fig. 6 shows 70 eV electron impact mass spectra of components X1, X2, and of a glucosamine standard. Glucosamine is an amino sugar that has a similar, 2-amino-2-deoxyhexose, structure and is an excellent model compound for elucidating the structure of components X1 and X2. The primary fragmentation of amino sugars is strongly influenced by the presence of the acetamido group[20,21], as shown here for glucosamine:




These two primary fragments break down further by elimination of acetic acid, acetamide, and ketene. The mass spectrum of glucosamine thus has peaks at 360 (primary fragment), 318 (loss of 42 from 360), 300 (loss of 60), 259 (loss of 59 and 42), 258 (loss of 60 and 42), 144 (primary fragment), 102 (loss of 42 from 144), and 84 (loss of 60). The spectra of components X1 and X2 are similar to that of glucosamine and suggest the presence of an acetamido group on the number 2 carbon of the alditol (which could be either the number 2 or the number 5 carbon of the sugar). Ions of

mass 144 and 302 may be assigned to the primary fragments:




The 144 fragment gives rise to the peaks at 102 and 84 as in the case of glucosamine. The ion of mass 302 produces secondary fragments at 260 (loss of 42), 242 (loss of 60), and 201 (loss of 59 and 42), and 200 (loss of 60 and 42), similar to the fragments arising from the 360 ions of glucosamine. The observed shift in mass of 58 units (360 − 302) indicates the replacement of acetate with hydrogen on one of the carbon atoms.

In order to gain additional information on the location of the amine group in the original sugar, a sample of *F. bozemanae* was prepared using sodium borodeu-

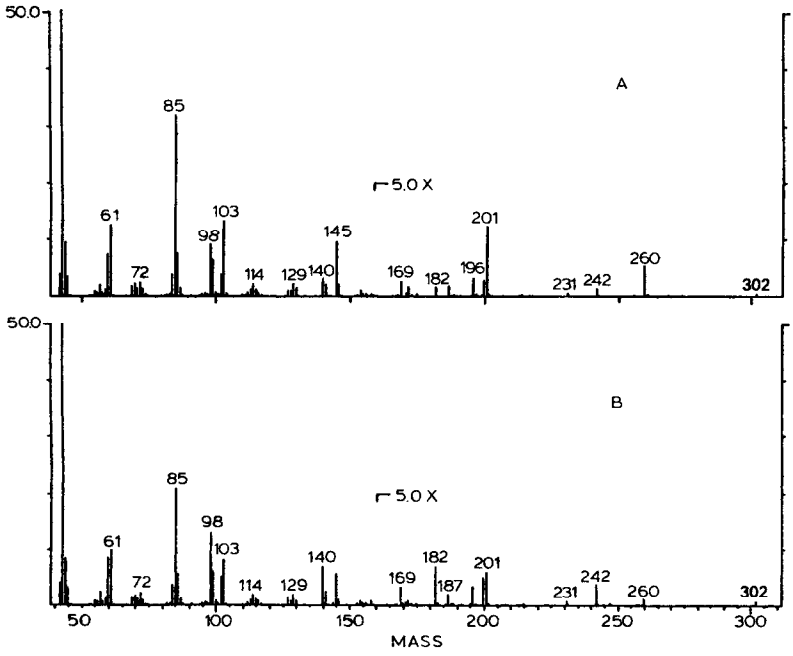
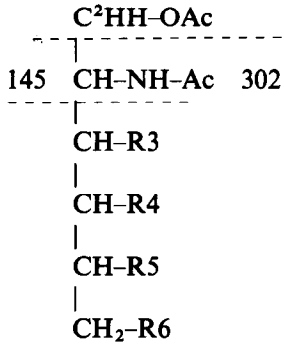


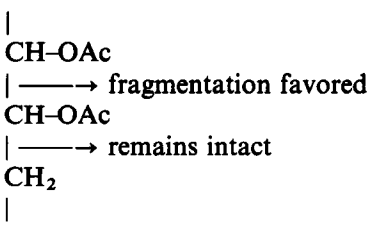

Fig. 7. Electron impact (70 eV) mass spectra of deuterated alditol acetates (A) X1 and (B) X2 from *F. bozemanae*. The heights of peaks in the mass spectra are multiplied by 2 from mass 40–160 and by 10 from mass 160–310.

teride to reduce the unknown sugars to deuterated alditols. The 70-eV electron impact spectra of resulting deuterated alditol acetates of components X1 and X2 are shown in Fig. 7. Ions at 85, 103 and 145 show the addition of deuterium to carbon 1.




This experiment demonstrates that the original X1 and X2 compounds were aldoses as opposed to polyalcohols which would not have been reduced by sodium borodeuteride. This experiment additionally proves that the amino group is on the number 2 carbon of the aldose.

The question still remains as to the location of the second deoxy carbon. Normally, a deoxy carbon can be located by the fragmentation pattern because the bonds adjacent to this carbon tend to remain intact[20,21] because the deoxy radical is less stable:




In the case of the components X1 and X2, fragmentation adjacent to the acetamido group dominates the spectra making it difficult to gain additional information regarding the position of the deoxy group. The fact that the mass 144 peak in the spectra of X1 and X2 is the same size as that in the spectra of glucosamine suggests the substituent group on the number 3 carbon (R3) is an acetate group because a hydrogen at that position would probably result in less fragmentation. This tentative assignment is speculative, however, without the aid of better model compounds.

Because electron impact mass spectrometry did not provide a molecular ion peak indicating the correct molecular weight of the X1 and X2 sugars, further confirmation of the structure assignment for components X1 and X2 was obtained by methane chemical ionization mass spectrometry. Fig. 8 shows methane CI spectra of component X1 and of a glucosamine standard. The spectrum of component X2 (not shown) was identical to that of component X1. The X1 and glucosamine spectra are quite similar except for a shift in mass of 58 a.m.u. The peak at mass 376 in the X1 spectrum represents the protonated molecular ion (M + H). The loss of acetic acid

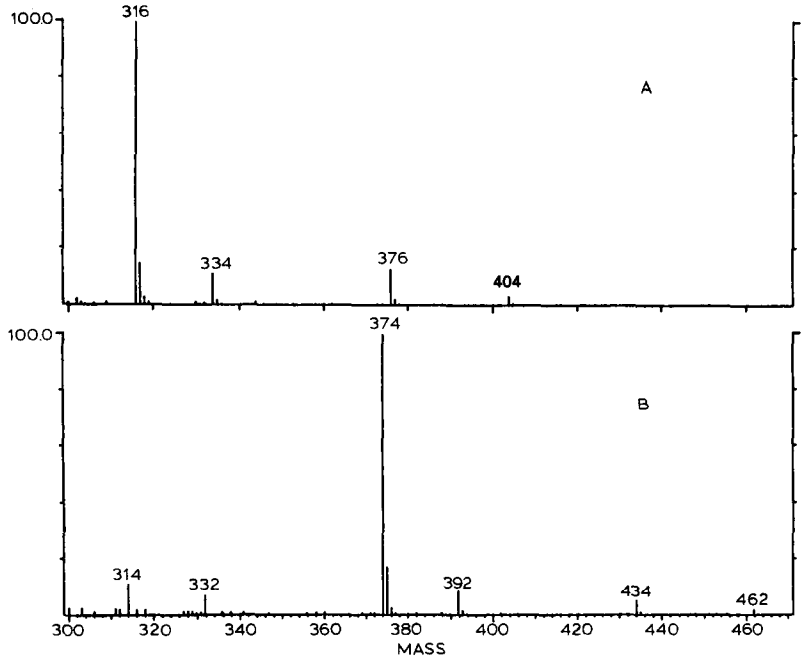



Fig. 8. Methane chemical ionization mass spectra of the alditol acetates of sugars (A) X1 and (B) glucosamine from *F. bozemanae*.

(M + H − 60) and ketene (M + H − 42) are observed at mass 316 and 334 respectively. Finally, a peak is also observed for the addition of $C_2H_5$ (M + 29) at mass 404. It should be noted that although we did observe the (M + H) peak in the spectra of these amino sugars, we did not see such a peak in the methane CI of neutral sugars; this agrees with previous reports[22,23] that neutral sugars do not give an (M + H) peak with methane CI and therefore isobutane or ammonia are preferred reagent gases for the alditol acetates of neutral sugars.

*The detection of heptose in Gram-negative organisms*

Fig. 9 presents a comparison of high resolution capillary chromatograms of the alditol acetates from whole cell samples of two different microorganisms: (A) a Gram-positive organism, *S. pyogenes,* and (B) a Gram-negative organism, *E. coli.* These two selected chromatograms again demonstrate the ability of capillary GC–MS to profile carbohydrate differences between bacteria. Heptose was readily detected in the chromatogram of the Gram-negative bacterium, but not in the chromatogram of the Gram-positive bacterium. Although background peaks were observed in the heptose region of the chromatogram of the Gram-positive organism, mass spectrometry showed that these peaks were not heptose. Fig. 10 compares the mass spectra of heptose identified in *E. coli* to an authentic heptose standard. The presence of the heptose peak is significant because this sugar is a distinct component of the lipopolysaccharide (LPS) of most Gram-negative bacteria. In addition to their staining characteristics, legionellae have been classified as Gram-negative organisms by electron microscopy[24,25] and by the isolation and identification of LPS[26]. How-

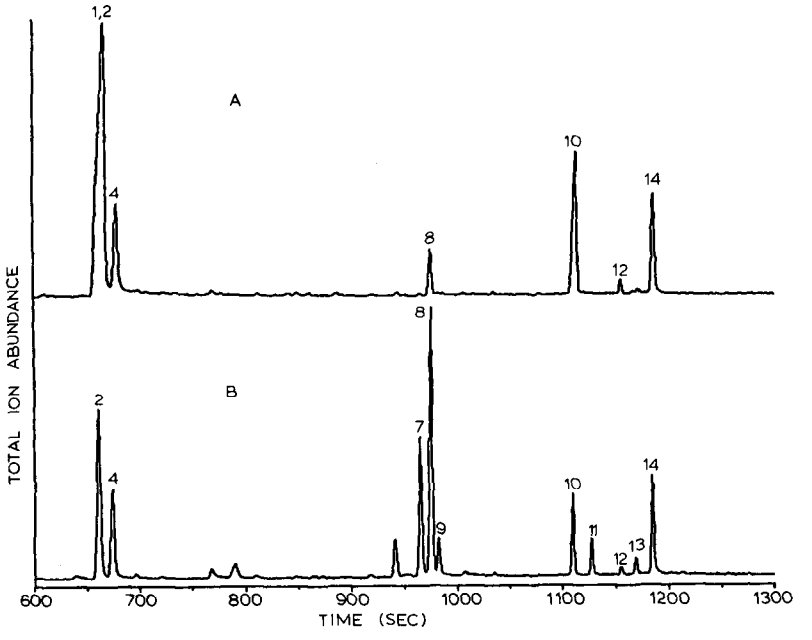



Fig. 9. Reconstructed total ion abundance chromatograms of the alditol acetates from (A) *S. pyogenes*, and (B) *E. coli*. Peak identities are: 1 = rhamnose; 2 = ribose; 4 = arabinose (neutral sugar internal standard); 7 = mannose; 8 = glucose; 9 = galactose; 10 = glucosamine; 11 = galactosamine; 12 = muramic acid; 13 = heptose; 14 = methyl glucamine (amino sugar internal standard).

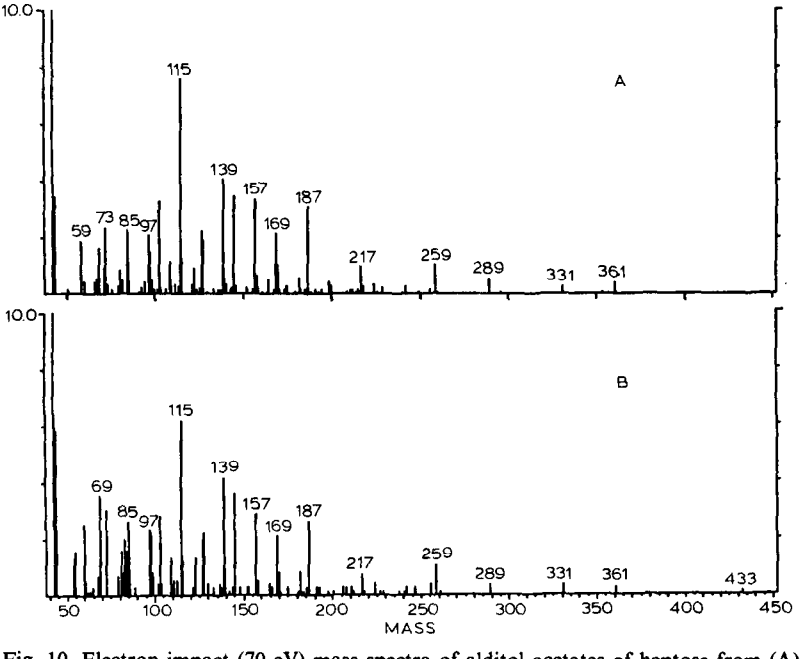



Fig. 10. Electron impact (70 eV) mass spectra of alditol acetates of heptose from (A) *E. coli* and (B) a heptose standard. The heights of peaks in the mass spectra are multiplied by 10.

ever, we have not been able to detect heptose in the whole *Legionella* samples that we have analyzed by GC–MS. Our ability to identify heptose in the chromatogram of *E. coli* (a Gram-negative organism) indicates that the absence of heptose in the *Legionella* chromatograms is not due to its loss in the hydrolysis and derivatization procedure, although it is possible that heptose is present below the detection limits of our method. We have detected heptose in LPS isolated from *Salmonella* and from *P. multocida*. Efforts to isolate LPS from Legionella and to analyze these samples for heptose are in progress in our laboratory.

## CONCLUSIONS

This work demonstrates the potential of capillary GC–MS in the direct chemical characterization of microorganisms. The methodology may be used to differentiate bacterial species or, alternatively, may be used to make taxonomic distinctions based upon chemical structure. The identification of chemical markers, indicative of specific chemical structures in the microorganisms can serve as steps in the identification of unknown bacteria. For example, muramic acid as a marker for peptidoglycan denotes the presence of bacteria, while the detection of heptose may differentiate many Gram-negative from Gram-positive bacteria. Other unique components such as ketodeoxyoctonic acid, tuberculostearic acid, mycolic acids, or the amino dideoxyhexoses found in some *Legionella* may also aid in bacterial classification. The logical extension of these concepts could lead to the development of specific trace methods for the detection of chemical markers in infected body fluids and tissues to indicate the presence of pathogenic microorganisms.

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