The Distribution of Heptose and 2-Keto-3-deoxy-octonate in Bacteroidaceae

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SUMMARY

A survey of phenol–water extracts of 17 different *Fusobacterium* and *Bacteroides* species, including subspecies, and of *Leptotrichia buccalis* has been carried out using colorimetric and chromatographic techniques to demonstrate the presence of heptose and 2-keto-3-deoxy-octonate. Both sugars were found in the water phase of the extracts from *Fusobacterium* strains and *Leptotrichia buccalis*. Neither heptose nor 2-keto-3-deoxy-octonate were detected in the water or the phenol phase of extracts from strains classified as *Bacteroides*.

INTRODUCTION

Generic classification of anaerobic Gram-negative non-sporeforming bacilli depends primarily on metabolic activities (*Anaerobe Laboratory Manual*, 1972; Subcommittee, 1970). *Fusobacterium*, including species formerly classified as *Sphaerophorus* (*Bergey's Manual of Determinative Bacteriology*, 1957), produces large amounts of butyric acid from glucose while most *Bacteroides* species do not. Some *B. melaninogenicus* strains ferment certain amino acids with formation of butyric acid, but in addition isobutyric, isovaleric and propionic acids are produced (Wahren & Gibbons, 1970). The same four fatty acids are also present in cultures of *B. praeacutus* and *B. putredinum*. *Leptotrichia buccalis* ferments glucose and a number of other carbohydrates with the formation of lactic acid.

Endotoxic lipopolysaccharide (LPS) has been prepared in this laboratory from oral strains of *Fusobacterium* (Kristoffersen & Hofstad, 1970) and *Bacteroides melaninogenicus* (Hofstad, 1968; Hofstad & Kristoffersen, 1971*a*), from a single strain of *B. fragilis* (Hofstad & Kristoffersen, 1970*a*) and from three strains classified as *Sphaerophorus necrophorus* (Hofstad & Kristoffersen, 1971*b*). Lipopolysaccharide from *Fusobacterium* and from one of the three *S. necrophorus* strains contained heptose and small amounts of 2-keto-3-deoxy-octonate (KDO). These sugars were not detected in LPS isolated from *B. melaninogenicus*, from the *B. fragilis* strain or from the remaining two strains of *S. necrophorus* (ATCC12290 and Institut Pasteur 3328), which, however, were later shown to belong to *Bacteroides* (Werner, 1970; Hofstad, unpublished). When present in LPS, heptose is a constituent of the basic polysaccharide core (Lüderitz, Staub & Westphal, 1966), while KDO provides the bridge between this core and lipid A (Osborn, 1963). These studies thus suggested a difference in wall chemistry between *Fusobacterium* and *Bacteroides* which might serve as a useful taxonomic criterion. Several other well-characterized strains representing separate *Fusobacterium* and *Bacteroides* species as well as two strains of *Leptotrichia buccalis* have therefore been examined for the presence of heptose and KDO.

Table 1. Distribution of heptose in phenol-water extracts of *Bacteroides*, *Fusobacterium* and *Leptotrichia buccalis*

Species	Strain	Water phase			Phenol phase		
		Dische's reaction	Chromatography		Dische's reaction	Chromatography	
			Paper	GLC		Paper	GLC
<i>B. coagulans</i>	VPI3476	—	—	—	+	—	
<i>B. fragilis</i>							
ss. <i>fragilis</i>	Lille E323	—	—		+	—	
ss. <i>fragilis</i>	NCTC9343	—	—	—	+	—	—
ss. <i>fragilis</i>	VPI5807	—	—		+	—	
ss. <i>distasonis</i>	ATCC8503	—	—		+	—	
ss. <i>thetaiotaomicron</i>	ATCC 12290	—	—		+	—	
ss. <i>thetaiotaomicron</i>	Lille 59	—	—		+	—	
ss. <i>thetaiotaomicron</i>	VPI5333	—	—		+	—	
ss. <i>vulgatus</i>	ATCC8482	—	—		+	—	
<i>B. furcosus</i>	VPI3255	—	—		+	—	
<i>B. hypermegas</i>	VPI2366 = ATCC25560	—	—		+	—	
<i>B. melaninogenicus</i>							
ss. <i>intermedius</i>	VPI4197 = ATCC25611	—	—	—	+	—	—
ss. <i>intermedius</i>	VPI7791C	—	—		+	—	
ss. <i>melaninogenicus</i>	VPI2381 = ATCC25845	—	—		+	—	
ss. <i>melaninogenicus</i>	VPI4196 = ATCC25847	—	—	—	+	—	—
ss. <i>asaccharolyticus</i>	VPI4199 = ATCC25846	—	—	—	+	—	—
<i>B. oralis</i>							
ss. <i>oralis</i>	VPI5832	—	—		+	—	
ss. <i>oralis</i>	VPI7570A	—	—		+	—	
<i>B. putredinum</i>	VPI4998-I	—	—	—	+	—	
<i>B. preacutus</i>	VPI0217-I	—	—		+	—	
<i>B. serpens</i>	VPI0950-I	—	—		+	—	
<i>F. mortiferum</i>	VPI0473 = ATCC9817	+	+				
<i>F. naviforme</i>	VPI4877	+	+				
<i>F. necrogenes</i>	VPI2368 = ATCC25556	+	+				
<i>F. necrophorum</i>	VPI6161	+	+				
<i>F. nucleatum</i>	VPI4355 = ATCC25586	+	+				
<i>F. nucleatum</i>	ATCC10953	+	+				
<i>F. plauti</i>	VPI4145	+	+				
<i>F. russi</i>	VPI0307	+	+				
<i>F. varium</i>	VPI0499A	+	+				
<i>F. varium</i>	ATCC8501	+	+				
<i>L. buccalis</i>	L 11 *	+	+				
<i>L. buccalis</i>	L66*	+	+				

* Own isolates.

METHODS

Organisms. The strains examined (Table 1) were obtained from: American Type Culture Collection, Rockville, Maryland, U.S.A. (ATCC); Institut Pasteur de Lille, France (Lille); National Collection of Type Cultures, Central Public Health Laboratory, London (NCTC); and the Virginia Polytechnic Institute and State University, Blacksburg, Virginia, U.S.A. (VPI).

Cultural conditions. Cultures were grown in 500 or 1000 ml screw-cap bottles filled to the top with the following medium (g/l): whale meat extract (Rieber & Søn A/S, Bergen, Norway), 10; proteose peptone (Oxoid), 15; NaCl, 5; KH_2PO_4 , 1.5; $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 3.5;

(NH₄)₂SO₄, 0.5; yeast extract (Oxoid), 3; L-cysteine HCl, 1; and glucose, 5; pH 7.0. For *Bacteroides oralis*, *B. melaninogenicus* and *Leptotrichia buccalis* the medium was supplemented with 5 % horse serum. The organisms were harvested by centrifuging cultures in the stationary phase of growth (after 1 to 3 days at 37 °C).

Extraction and purification methods. Washed organisms were extracted with constant stirring with 45 % aqueous phenol for 15 min at room temperature (approx. 22 °C). After centrifuging for 30 min at 2500 g the water phase was pipetted off, dialysed against tap water and freeze-dried. Lipopolysaccharide was purified from the water phase of some strains by ultracentrifuging (100 000 g for 60 min), and treatment with deoxyribonuclease and ribonuclease (Hofstad & Kristoffersen, 1970a). The phenol phase was freed from particulate materials by addition of an equal volume of 90 % phenol and centrifuging (12 000 g for 30 min). Three volumes of sodium acetate-methanol (1 ml of saturated sodium acetate in 100 ml of methanol) were then added to the cleared phenol phase, and the mixture left at 4 °C overnight. The precipitate was centrifuged, taken up with water and dialysed. Following dialysis for 4 days against tap water the suspension within the dialysis bag was freeze-dried without further treatment.

Colorimetric determinations. Heptose was estimated by the sulphuric acid cysteine reaction as described by Dische (1953), or with the modifications of Osborn (1963) or Wright & Rebers (1972). The absorbance of the reaction mixture was examined over the range 450 to 600 nm. A peak or a shoulder at 500 to 510 nm was taken as a qualitative indication of heptose. The presence of KDO was investigated by the thiobarbituric acid method (Weissbach & Hurwitz, 1959). The absorbance was examined over the range of 500 to 600 nm, and a peak or a shoulder at 550 nm recorded as a positive reaction. Purified LPS from *Salmonella typhi* 0901 (Difco Laboratories, Detroit, Michigan, U.S.A.) and *Veillonella* (Hofstad & Kristoffersen, 1970b) were used as positive controls for heptose and KDO. The absorption spectra were recorded in a Unicam SP 800 spectrophotometer.

Paper chromatography. For demonstration of heptose samples were hydrolysed with 3 M-HCl for 3 h in an atmosphere of nitrogen at 100 °C. After removal of acid *in vacuo* at room temperature (approx. 22 °C) in the presence of NaOH pellets, the samples were subjected to circular chromatography on Whatman no. 1 paper with *n*-butanol:pyridine:water (6:4:3 by vol.) and the dried chromatograms stained with aniline hydrogen phthalate (Partridge, 1949). 2-Keto-3-deoxy-octonate was sought in samples hydrolysed with 0.05 M-H₂SO₄ for 30 min and desalted by neutralizing with Ba(OH)₂ followed by centrifuging and evaporation of the supernatant to dryness *in vacuo*. Chromatograms were run with *n*-butanol:pyridine:0.1 M-HCl (5:3:2 by vol.) as solvent. The thiobarbituric acid reagent of Warren (1960) was used to detect KDO. *Salmonella typhi* 0901 LPS was included as a positive control.

Gas-liquid chromatography (GLC). Samples hydrolysed in 0.1 M-HCl at 100 °C for 48 h were neutralized with Amberlite IRA 410, HCO₃⁻ form, and the aldoses converted to alditol acetates by the method of Sawardeker, Sloneker & Jeanes (1965). Gas-liquid chromatography was run in a Varian aerograph, Model 1520B with a flame ionization detector, and fitted with a glass column (0.32 × 152 cm) packed with 3 % ECNSS-M (w/w) on Chromosorb Q, 100 to 120 mesh. The flow of gas (N₂) was 30 ml/h and the detector temperature 200 to 210 °C. Samples (1 µl) of the alditol acetates in chloroform were injected. After elution of glucitol the initial isotherm column temperature of 170 °C was raised to 190 °C.

Fig. 1. Absorption spectra in the Dische reaction of phenol phases from *Staphylococcus* NRRLB2628 (1), *Bacteroides putredinium* VPI4899-1 (2), *Bacteroides furcosus* VPI3255 (3), and *Lactobacillus* La61 (4).

RESULTS

Heptose

In pilot experiments the absorption spectra given by samples of freeze-dried water phases from strains of *Bacteroides* and *Fusobacterium* in the Dische reaction for heptose and in the modified reactions were compared. The absorbance at 500 to 510 nm, corresponding to heptose, was highest in the modified reactions, but tended to be obscured by the very high absorption at 400 to 450 nm given by 6-deoxysugars, which were present in most preparations. The procedure of Dische was therefore used in the final experiments.

Heptose was not detected in 1 to 2 mg samples of freeze-dried water phases obtained by phenol water extractions of *Bacteroides* strains (Table 1), nor in purified LPS. All the *Bacteroides* phenol phases gave an absorption peak at 500 to 505 nm in the sulphuric acid-cysteine reaction of Dische. However heptose was not detected by paper chromatography of acid hydrolysates of the same phenol phases. Two strains of *Lactobacillus*, isolated from human saliva, and the *Staphylococcus* strain NRRLB2628 were then extracted with phenol-water. The phenol phases from all three strains gave an absorption peak at 500 to 505 nm in the Dische reaction (Fig. 1), but heptose could not be demonstrated by paper chromatography. Finally, water and phenol phases from some *Bacteroides* strains were examined for neutral sugars by GLC. No trace of heptose was observed. The neutral sugars found were the same as those demonstrated by paper chromatography, namely galactose, glucose, fucose, rhamnose, trace amounts of mannose, and (in the water phase) ribose. *Veillonella* LPS was used as a positive control for heptose.

Water phases obtained from the *Fusobacterium* strains and from the two strains of *Leptotrichia buccalis* contained heptose. On chromatograms stained with aniline hydrogen phthalate the characteristic red-brown band corresponding to the heptose was located between the galactose and glucose bands.

2-Keto-3-deoxy-octonate

2-Keto-3-deoxy-octonate could not be demonstrated by the thiobarbituric acid reaction (3 mg samples) or by paper chromatography (2 mg samples) in water phases obtained by extraction of the *Bacteroides* strains (Table 2) or in purified LPS. Freeze-dried water phases from *Bacteroides* strains VPI0950-1 and VPI2368 produced a peak at 540 to 545 nm in the thiobarbituric acid reaction. Water phases from all *Bacteroides* strains gave an absorption peak at 530 to 535 nm. None of the phenol phases (5 mg samples) reacted in the thiobarbituric acid reaction.

Table 2. *Distribution of KDO in phenol-water extracts of Bacteroides, Fusobacterium and Leptotrichia buccalis*

Species	Strain	Water phase		Phenol phase
		Thiobarbituric acid reaction	Paper chromatography	Thiobarbituric acid reaction
<i>Bacteroides</i> spp.	*	—	—	—
<i>F. mortiferum</i>	VPI0473 = ATCC9817	+	+	
<i>F. naviforme</i>	VPI4877	+	+	
<i>F. necrogenes</i>	VPI2368 = ATCC25556	—	+	
<i>F. necrophorum</i>	VPI6161	+	+	
<i>F. nucleatum</i>	VPI4355 = ATCC25586	+	+	
<i>F. nucleatum</i>	ATCC10953	+	+	
<i>F. plauti</i>	VPI4145	+	+	
<i>F. russi</i>	VPI0307	+	+	
<i>F. varium</i>	VPI0499-A	+	+	
<i>F. varium</i>	ATCC8501	—	+	
<i>L. buccalis</i>	L11†	+	+	
<i>L. buccalis</i>	L66†	+	+	

* Same strains as listed in Table 1.

† Own isolates.

Paper chromatograms of acid hydrolysates of the water phases from *Fusobacterium* strains gave a faint band with all strains, which had the same *R* value as KDO released from *S. typhi* 0901 LPS, hydrolysed in the same way. A peak at 550 nm was not detected in the thiobarbituric acid reaction with the water phases from strains VPI2366 and ATCC8501. The peak produced by the other *Fusobacterium* strains was very small compared with the peak given by similar amounts of *Veillonella* LPS or water phases from the two *Leptotrichia buccalis* strains.

DISCUSSION

The outstanding feature of this study was the absence of heptose in phenol-water extracts of strains classified as *Bacteroides*. Heptose is absent also from lipopolysaccharides isolated from *Neisseria catarrhalis* (Adams, Tornabene & Yaguchi, 1969), *N. sicca* (Adams, 1971), *Moraxella duplex* and *Acinetobacter calcoaceticus* (Adams, Quadling, Yaguchi & Tornabene, 1970), *Pseudomonas pavonacea* (Wilkinson, Galbraith & Lightfoot, 1973), *Xanthomonas* species (Volk, 1966) and myxobacteria (Rosenfelder, Lüderitz & Westphal, 1974). In contrast, heptose was present in all strains of *Fusobacterium* examined and in *Leptotrichia buccalis*.

The chromatographic migration rate of the heptose was similar to that reported for L-glycero-D-mannoheptose (or D-glycero-D-mannoheptose) (Davies, 1957), the aldohexose commonly present in bacterial lipopolysaccharides.

Paper chromatography of acid hydrolysates was superior to the sulphuric acid-cysteine reaction for the demonstration of heptose. When heptose was present in small amounts with other sugars its absorption peak at 505 nm tended to be lost between the absorptions at 400 to 450 nm and 520 to 550 nm produced by deoxysugars and large amounts of glucose, respectively. Furthermore, the sulphuric acid-cysteine reaction apparently is not specific for heptose as materials recovered in the phenol phase also by extraction of Gram-positive bacteria gave positive reactions.

Acid-labile KDO was detected with difficulty in extracts from the *Fusobacterium* strains. Although the amounts of KDO in the water phases or in purified LPS were not quantified,

comparison with *Salmonella typhi* 0901 and *Veillonella* LPS suggests that KDO is present in much smaller amounts in the LPS of *Fusobacterium*, as already reported by Kristoffersen & Hofstad (1970), than in the LPS prepared from most other Gram-negative bacteria. It is possible, however, that during mild acid hydrolysis some of the KDO present was converted to derivatives which did not react with thiobarbituric acid (Volk, Salomonsky & Hunt, 1972).

The *Bacteroides* species differed qualitatively from *Fusobacterium* species and *Leptotrichia buccalis* in containing no KDO in phenol-water extracts and LPS. KDO is absent from LPS isolated from *Neisseria catarrhalis* (Adams *et al.* 1969), *Moraxella duplex* and *Acinetobacter calcoaceticus* (Adams *et al.* 1970), *Vibrio cholerae* (Jackson & Redmond, 1971) and from some *Pseudomonas* species (Wilkinson *et al.* 1973).

Because of the absorption given by deoxysugars at 530 to 535 nm in the thiobarbituric acid reaction, paper chromatography seems to be better suited for detection of KDO when present in small amounts in mixture with these sugars.

This study corroborates the generic separation of *Fusobacterium* and *Bacteroides* and extends the definition of these two genera and of *Leptotrichia buccalis*. The inclusion of *Sphaerophorus* strains in genus *Fusobacterium* (Subcommittee, 1970) seems justified.

The taxonomy of anaerobic Gram-negative rods is at present based primarily on the production of fatty acids in glucose-containing media, and on other biochemical tests and susceptibility to antibiotics. Our findings show that analysis of wall components may also be used as a taxonomic criterion.

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