BACTERIAL CHARACTERISATION

Phenotypic characteristics and lipopolysaccharides of human and animal isolates of *Fusobacterium necrophorum*

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As part of a collaborative study, six culture collection isolates and 50 coded isolates of *Fusobacterium necrophorum* were examined for the types of lipopolysaccharides (LPS) they contained, and to see if this related to their reactions in a range of phenotypic tests and their susceptibility to a panel of six antimicrobial compounds. The biotype B type strain, putative biotype B isolates and human isolates were predominantly coccobacillary, had rough type LPS and some of these isolates (8 of 26) required incubation for >2 days to demonstrate lipase activity. The biotype A type strain, putative biotype AB isolates and most putative biotype A isolates (16 of 18) were predominantly rod-shaped, had either smooth LPS or low M_r rough type LPS and all demonstrated lipase activity within 2 days. The other two putative biotype A isolates were coccobacillary and had rough type LPS, and one of these required incubation for >2 days to demonstrate lipase activity. The results of these latter two isolates more closely resembled biotype B. A few isolates were asaccharolytic, but most fermented one or more of glucose, fructose, maltose and galactose. There was no correlation between fermentation pattern and LPS type, biotype or source of isolate (animal or human) but, with the exception of two aberrant isolates, there was good correlation between cellular morphology, type of growth in liquid media and LPS type.

Introduction

The anaerobic gram-negative bacterium *Fusobacterium necrophorum* is pathogenic for man and animals [1–5]. Previously it had been classified in three distinct species, *Sphaerophorus necrophorus*, *S. funduliforme* and *S. pseudonecrophorus* [6] and later as biotypes A, B and C, respectively, of *F. necrophorum* [7]. Biotype C was reclassified as *F. pseudonecrophorum* on the basis of DNA homology studies, and shown to differ from biotypes A and B by its inability to produce lipase, haemolysis and haemagglutination, and was not associated with liver abscess formation in animals [8]. More recently, *F. pseudonecrophorum* was shown to be the same as *F. varium* and the latter name has been adopted for these strains [9]. Biotypes A and B were reclassified as *F. necrophorum* subsp. *necrophorum* and *F. necrophorum* subsp. *funduliforme*, respectively, on

the basis of DNA homology studies, and shown to differ from each other in tests for haemagglutination, lipase production and mouse lethality [10], and lipopolysaccharides (LPS) extracted from these organisms were shown to differ in their chemical composition and biological properties [11].

There is disagreement between published results for fermentation patterns and lipase activity of these organisms. The aim of this part of a collaborative study of human and animal isolates was to examine the LPS type, to see if this correlated with the phenotypic characteristics and antibiotic susceptibility of isolates of *F. necrophorum* and to compare these results with the stated putative biotypes.

Materials and methods

Bacterial strains

F. necrophorum subsp. *necrophorum* JCM 3718^T, *F. necrophorum* subsp. *funduliforme* JCM 3724^T and *F. pseudonecrophorum* JCM 3722^T (*F. varium*) were obtained from the Japanese Collection of Microorganisms, Wako-shi, Saitama, Japan. *F. necrophorum*

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NCTC 10575, 10576 and 10577 were obtained from the National Collection of Type Cultures, 61 Colindale Avenue, London. Fifty isolates of *F. necrophorum* (including four duplicate pairs, one pair being duplicates of NCTC 10575) were obtained from Dr J. Brazier, PHLS Anaerobe Reference Unit, Cardiff, as part of a collaborative study; these were received coded F1–F50.

Identification

Strains were identified by standard phenotypic tests in anaerobe investigation medium (AIM), and gas chromatographic analysis of short-chain fatty-acid end-products of metabolism by the method of Brown *et al.* [12]. Cell morphology was examined in cultures grown in AIM-cooked meat broth (CMB). Incubation was at 37°C in an anaerobic cabinet (Don Whitley Scientific, Shipley, W. Yorkshire) with an atmosphere of H₂ 10%, CO₂ 10%, N₂ 80%.

Extraction of LPS

This was slightly modified from the method of Fomsgaard *et al.* [13]. The organisms were grown anaerobically for 22–24 h in 20 ml of AIM supplemented with glucose 5 g/L. Cells were harvested by centrifugation at 4000 *g* for 20 min and resuspended in 500 µl of pyrogen-free de-ionised water. To the suspended cells, 500 µl of phenol 90% w/w aqueous solution were added and, after vortex mixing for 10 s, the suspension was heated at 70°C for 10 min. After cooling in an ice bath for at least 2 min and centrifugation at 3000 *g* for 4 min, the clear upper layer was collected and stored at –20°C.

Polyacrylamide gel electrophoresis

LPS extract was mixed with an equal volume of SDS-PAGE double strength sample buffer and 25-µl volumes were run on acrylamide 12% slab gels with the buffer system of Laemmli [14], but excluding SDS. The gels were silver stained by the method of Tsai and Frasch [15], as modified by Hancock and Poxton [16].

Antibiotic susceptibility

MIC tests were done on Diagnostic Sensitivity Test Agar (Oxoid) supplemented with horse blood 10% v/v and

containing two-fold dilutions of antibiotics. The antibiotics tested were metronidazole, co-amoxiclav, imipenem, clindamycin, sparfloxacin and cefoxitin. Plates were inoculated with *c.* 10⁵ cfu by a multipoint inoculator (Mast Laboratories) and incubated anaerobically for 48 h. MIC range, MIC50 and MIC90 were calculated.

Results

The coded isolates contained four duplicate pairs, and one of those pairs was the same strain as NCTC 10575. Duplicates within each set gave identical results, except that the authors' isolate of NCTC 10575 failed to lower the pH of any of the test sugars by >0.3 pH units below that of the carbohydrate-free control culture, whereas both of the duplicates showed a pH fall of 0.6 or 0.8 with both glucose and fructose. However, all three isolates were considered to be asaccharolytic. The results obtained with only one isolate of each duplicate (or triplicate) set are included in the following sections.

Identification of isolates

All isolates were anaerobic, gram-negative, non-motile rods or coccobacilli that produced major amounts of butyric acid and smaller amounts of acetic and propionic acids as end-products of metabolism. Some isolates also produced varying amounts of lactic acid and some produced trace amounts of succinic acid. All isolates converted threonine to propionate and produced indole. With the exception of *F. pseudonecrophorum* JCM 3722, all isolates converted lactate to propionate, did not hydrolyse aesculin, reduce nitrate or digest gelatin, failed to grow or grew poorly in the presence of bile, and produced lipase, although the time taken to detect this effect varied between isolates. *F. pseudonecrophorum* JCM 3722 did not convert lactate to propionate, hydrolysed aesculin, its growth was stimulated in the presence of bile, it did not produce lipase and was the only isolate to ferment mannose. No isolates fermented lactose, sucrose, trehalose or cellobiose, and fermentation of glucose, maltose, fructose and galactose varied between isolates. Isolates could be grouped by a combination of their reactions in converting lactate to propionate and the time taken to detect lipase production (Table 1), or by their fermentation patterns (Table 2).

Table 1. Distribution of culture collection strains and putative biotypes by the ability to convert lactate to propionate and time taken to detect lipase activity

Pattern	Lactate to propionate	Lipase detected by		Number of isolates	Putative biotype (n)				JCM no.	NCTC no.
		2 days	3–7 days		A	AB	B	Human		
a	+	+	...	41	17	2	3	15	3718*	10575, 10576, 10577
b	+	–	+	9	1	0	1	6	3724†	...
c	–	–	–	1	0	0	0	0	3722‡	...

**F. necrophorum* biotype A.

†*F. necrophorum* biotype B.

‡*F. pseudonecrophorum* (*F. varium*).

Table 2. Distribution of culture collection strains and putative biotypes by their fermentation patterns

Pattern	Fermentation of					Number of isolates	Putative biotype (n)				JCM no.	NCTC no.
	glucose	maltose	fructose	galactose	mannose		A	AB	B	Human		
1	+	+	+	+	—	11	9	0	2	0	...	...
2	+	+	+	—	—	13	1	0	0	11	...	10576
3	+	+	—	+	—	3	2	0	0	1	...	...
4	+	—	+	+	—	3	2	0	1	0	...	...
5	+	+	—	—	—	4	0	0	1	3	...	...
6	+	—	+	—	—	3	1	0	0	0	3718*, 3724†	...
7	+	—	—	+	—	3	0	2	0	0	...	10577
8	+	—	—	—	—	7	2	0	0	5	...	...
9	—	—	—	—	—	3	1	0	0	1	...	10575
10	+	—	+	—	+	1	0	0	0	0	3722‡	...

**F. necrophorum* biotype A.†*F. necrophorum* biotype B.‡*F. pseudonecrophorum* (*F. varium*).

The cellular morphology in liquid culture was either predominantly coccobacillary, with the cells growing in long, meandering chains and in clumps that formed a dense floccular pellet with a virtually clear supernate, or they were predominantly slender rods and filaments that formed an even turbidity throughout the medium with or without some pellet. One isolate (F37) was an exception in that it was predominantly in clumps of filamentous rods after incubation for 24 h, but changed to coccobacilli after 48 h, growth appearing as a dense floccular pellet at both times.

Examination of LPS profiles

Three types of LPS patterns were demonstrated (Fig. 1). A high M_r ladder pattern, typical of smooth LPS, was produced by 11 isolates, including *F. necrophorum* subsp. *necrophorum* JCM 3718 and *F. necrophorum* NCTC 10577. A rough LPS pattern comprising a large low M_r mass band was seen in 29 isolates, including *F. necrophorum* subsp. *funduliforme* JCM 3724 and *F. pseudonecrophorum* JCM 3722. The remaining 11 isolates, including *F. necrophorum* NCTC 10575 and 10576, produced profiles that comprised only small bands of weakly staining low M_r material; similar

Fig. 1. Silver stain of LPS of *F. necrophorum*. Lanes 1–5, smooth LPS (biotype A and putative biotype A strains JCM 3718, NCTC 10577, F3, F20 and F45); 6–10, rough LPS (biotype B, putative biotype B and human strains JCM 3724, F1, F4, F19 and F35); 11–15, low M_r LPS (biotype A and putative biotype A and AB strains NCTC 10575, F5, F16, F24 and F46).

results were obtained from repeated extractions of these isolates even when gels were loaded with increased sample volumes. In some gels a band with a relative mobility of 60% appears to be common to most of the LPS extracts. However, this band was also found in phenol extracts when sterile culture medium was processed identically to cultures, and probably represents contamination of the extracts. Table 3 shows the distribution of biotypes by LPS type and cell morphology, and Table 4 shows the distribution of each test isolate by group, fermentation pattern, cellular morphology and LPS type. *F. pseudonecrophorum* was rod-shaped and had a rough type LPS. The other 22 isolates that were predominantly rod-shaped in films from CMB had either smooth LPS patterns or small bands of low M_r rough types of LPS and comprised *F. necrophorum* subsp. *necrophorum* JCM 3718, *F. necrophorum* NCTC 10575, 10576 and 10577, 16 putative biotype A and two putative biotype AB isolates. All 28 isolates that were predominantly coccoid or coccobacillary in films from CMB had rough type LPS and comprised *F. necrophorum* subsp.

funduliforme JCM 3724, two putative biotype A (F31, F41), four putative biotype B, and 21 human isolates. All nine isolates that required incubation for > 2 days on egg-yolk agar to show lipase activity (i.e., group b) were coccobacillary and had rough type LPS. This group comprised *F. necrophorum* subsp. *funduliforme* JCM 3724, one putative biotype A (F41), one putative biotype B and six human isolates.

Antibiotic susceptibility

Table 5 shows the results of MIC tests done on *F. necrophorum* NCTC 10575, 10576 and 10577, *F. necrophorum* subsp. *funduliforme* JCM 3724, *F. pseudonecrophorum* JCM 3722, 43 other *F. necrophorum* isolates and the control organisms *Bacteroides fragilis* NCTC 9343 and *Clostridium perfringens* NCTC 8237. The MIC values for *F. pseudonecrophorum* JCM 3722 were obviously different from the other isolates; otherwise there was no correlation between susceptibility pattern and fermentation pattern, LPS type or biotype.

Table 3. Distribution of culture collection strains and putative biotypes by cellular morphology and LPS type

Cellular morphology	LPS type	Number of isolates	Putative biotype (n)				JCM no.	NCTC no.
			A	AB	B	Human		
Rods	Smooth	11	9	0	0	0	3718*	10577
	Rough	1	0	0	0	0	3722†	...
	Low M_r	11	7	2	0	0	...	10575, 10576
Coccoid	Smooth	0	0	0	0	0	...	...
	Rough	28	2	0	4	21	3724‡	...
	Low M_r	0	0	0	0	0	...	...

* *F. necrophorum* biotype A.

† *F. necrophorum* biotype B.

‡ *F. pseudonecrophorum* (*F. varium*).

Table 4. Distribution of isolates by group, fermentation pattern, cellular morphology and LPS type

Group	Fermentation pattern	Number of isolates	Number of isolates	Isolate F or culture collection number									
				Rods					Coccoid				
				LPS type					Number of isolates	LPS type			
				Smooth	Rough	Low M_r				Smooth	Rough	Low M_r	
a (45)	1	9	8	3/49 7 8	...	5 11 24 28 33	1	...	...	19/23	...	...	...
	2	7	2	38	...	10576*	5	...	...	2 6 21 37 50	...	...	...
	3	3	2	20 39	...	...	1	...	...	29	...	...	...
	4	3	2	36	...	25	1	...	...	1	...	...	...
	5	4	0	...	...	...	4	...	...	22 26 27 43	...	...	...
	6	2	2	34 3718†	...	...	0	...	...	...	...	...	...
	7	3	3	10577*	...	16 46	0	...	...	...	...	...	...
	8	7	1	45	...	...	6	...	12 13 15 31 35 42	...	...	...	...
	9	3	2	...	...	10/32/10575* 14	1	...	...	47	...	...	...
b (10)	1	2	0	...	...	...	2	...	...	30 41	...	...	...
	2	6	0	...	...	...	6	...	4 9 17/40 18 44 48	...	...	...	...
	6	1	0	...	...	...	1	...	...	3724‡	...	...	...
c (1)	10	1	1	...	3722§	...	0	...	...	...	...	...	...

/, Duplicates of the same strain, and counted as one isolate in the 'Number of isolates' columns.

* *F. necrophorum* NCTC isolates.

† *F. necrophorum* biotype A, JCM isolate.

‡ *F. necrophorum* biotype B, JCM isolate.

§ *F. pseudonecrophorum* (*F. varium*), JCM isolate.

Table 5. MIC range, MIC50 and MIC90 of 47 *F. necrophorum* isolates to six antimicrobial compounds

Species	Strain no.	MIC (mg/L) of						
			co-amoxiclav	cefoxitin	clindamycin	imipenem	metronidazole	sparfloxacin
<i>F. necrophorum</i>	(47 isolates)	Range	≤ 0.01–0.25	≤ 0.06–0.25	0.06–0.25	≤ 0.008–0.06	0.06–0.5	0.25–4
		MIC50	0.06	≤ 0.06	0.12	0.01	0.12	2
		MIC90	0.12	0.12	0.12	0.03	0.25	2
<i>F. pseudonecrophorum</i>	JCM 3722		2	16	0.5	8	0.25	8
<i>B. fragilis</i>	NCTC 9343		1	4	0.06	0.25	0.5	1
<i>C. perfringens</i>	NCTC 8237		≤ 0.06	≤ 0.5	0.06	0.06	1	0.25

Discussion

The results obtained here are in agreement with others [9] that *F. pseudonecrophorum* is a synonym for *F. varium*. *F. necrophorum* subsp. *necrophorum* (*F. necrophorum* biotype A) and *F. necrophorum* subsp. *funduliforme* (*F. necrophorum* biotype B) have been differentiated by various workers by colonial morphology [17–19], cell morphology [17–19], haemagglutination patterns [7, 17–19], lipase activity [8–10, 19], haemolysis [7, 17–19], and mouse lethality [18, 19]. There is good agreement in the published results for most of these tests, but lipase activity of biotype B has been reported as both positive [8, 9] and negative [10, 19]. In this study, excepting *F. pseudonecrophorum*, all isolates gave positive results for lipase activity although the biotype B type strain, one putative biotype B isolate, six human isolates and one putative biotype A isolate (F41) required incubation for ≥ 3 days to detect this. However, isolate F41 produced coccobacillary cells and a rough type LPS and more closely resembles biotype B than biotype A.

The fermentation of glucose, fructose, maltose, mannose and galactose has variably been reported for *F. necrophorum*. Conflicting reports on fermentation results probably arose because different methods were used to measure fermentation. Some researchers [20] interpret fermentation as being positive when the final pH value is below 5.5, weak between 5.5 and 6.0 and negative above that level, and report most strains of *F. necrophorum* as asaccharolytic but with some strains giving weak results with glucose and fructose. Another study [17] found the terminal pH range from glucose fermentation to be 5.35–6.1 and, in tests with bromothymol blue which would indicate acid at pH 6.0 and below, reported the majority of *F. necrophorum* isolates to ferment glucose, fructose, maltose, mannose and galactose after incubation for 4 days. In this study, fermentation was considered positive if the pH of the test carbohydrate culture was 1 pH unit or more below that of a carbohydrate-free culture (i.e., at least 10 times more acidic than control). After incubation for 2 days, the glucose fermentation pH ranged from 5.4 to 7.2 (average 5.79), with the pH of the majority (48 of 51) of isolates being from 5.55 to 5.85. Only three isolates – NCTC 10575, one putative biotype A (F14) and one human isolate (F47) – failed to ferment any of the test carbohydrates. Other isolates

of *F. necrophorum* fermented one or more of glucose, fructose, maltose and galactose, and only the *F. pseudonecrophorum* strain fermented mannose. There was no relationship between fermentation pattern and either animal biotype or human source, or LPS type.

In films from CMB cultures the cellular morphology was predominantly either coccobacillary or rods and filaments. All of the isolates that were coccobacillary had rough type LPS, and included the biotype B type strain, all four putative biotype B isolates, all 21 human isolates and two putative biotype A (F31, F41) isolates. These latter two isolates need to be tested for virulence and haemagglutination in order to re-assess their designation of biotype A.

Isolates that were rods and filaments demonstrated two distinct types of LPS: 1, a ladder pattern typical of smooth LPS, and included *F. necrophorum* JCM 3718^T, NCTC 10577 and nine putative biotype A isolates; 2, an LPS pattern that comprised only small amounts of low M_r material, and included *F. necrophorum* NCTC 10575, NCTC 10576, seven putative biotype A and two putative biotype AB isolates. This latter LPS pattern was also demonstrated in repeated extractions, even when the amount of sample run in gels was increased, suggesting a weak staining reaction with silver by its resistance to oxidation by periodate. With the exception of the two putative biotype A isolates (F31, F41) that were coccobacillary and had rough type LPS, there was good correlation between cellular morphology, biotype or species source, and LPS type.

Intraperitoneal inoculation of viable cells of *F. necrophorum* into mice showed biotype A isolates to be more lethal than those of biotypes B and AB. However, LPS extracted from the same strains showed only minor differences in endotoxicity between biotypes A and B when tested similarly in mice, whereas biotype AB was much less endotoxic [21]. In contrast, the LPS of *F. necrophorum* biotype A (VPI 2891, the parent strain of JCM 3718^T) has been shown to differ in its chemical composition and biological activity from that of biotype B [11]. The latter authors employed chick embryo lethality, local Shwartzman reaction and febrile responses in rabbits to demonstrate these differences, tests that are probably more sensitive than mouse lethality for detecting endotoxins.

In the present study, with the exception of isolates F31 and F41, the type strain and 10 putative biotype A isolates had smooth type LPS, whereas nine other putative biotype A and two putative biotype AB isolates had LPS comprising small amounts of low M_r material only. Biotyping of *F. necrophorum* is based on the results of tests for cellular morphology, sedimentation in broth culture, colonial morphology, direct haemagglutination, and virulence and pathogenicity [7, 17, 21]. Only the first two of these tests were done by the authors and, in the results obtained, the putative biotype AB isolates resembled biotype A. However, results of other tests done by our collaborators might show that the isolates with a low M_r rough type LPS form a cluster distinct from the type strain of biotype A.

It has been shown, with the exception of one aberrant strain (not included in this study), that human isolates resemble those of biotype B in being of low pathogenicity in mice [22]. In this study, the type strain (JCM 3724), all four putative biotype B and all 21 human isolates had a rough type LPS. This indicates that there may be a relationship between LPS type and virulence in isolates of *F. necrophorum*.

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