

An Identification Scheme for Oral Non-pigmented *Prevotella* (*Bacteroides*) Species

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Received 4 June 1991; revised 8 August 1991

Three approaches were used to characterise 13 reference strains of non-pigmented *Prevotella* (*Bacteroides*) spp. and 91 clinical isolates from 21 adult patients with periodontal diseases. Strains were examined in tests for: (1) biochemical and morphological characters comprising microscopic and colonial appearance, biochemical and fermentation activity, tolerance of bile salts and dyes, susceptibility to antibiotic disks and fluorescence under u.v. light; (2) production of volatile and non-volatile fatty acids by gas-liquid chromatography (GLC); and (3) preformed enzymes profiles. Species were distinguished by: hydrolysis of hippurate, starch and aesculin; fermentation of lactose, sucrose, rhamnose, xylose, arabinose, cellobiose, salicin, inulin and xylan; tolerance of Victoria blue 4R; and fluorescence. In GLC analysis, production of propionic, iso-butyric, iso-valeric and phenylacetic acids gave useful discrimination between species. Discriminatory enzyme profiles were obtained with tests for β -xylosidase, β -glucuronidase, β -glucosidase, α -fucosidase, β -*N*-acetylglucosaminidase and α -mannosidase. Correlation between the three approaches was good and an identification scheme was designed for non-pigmented *Prevotella* spp. Of the 91 clinical isolates from adult periodontal diseases, 63 gave results identical with those of reference strains and 21 showed only minor variation. The commonest species were *P. buccae* (42 isolates), *P. veroralis* (15 isolates) and *P. oralis* (eight isolates).

KEY WORDS—*Prevotella*; oral flora; periodontal disease; identification; gas-liquid chromatography; enzyme tests.

INTRODUCTION

The melaninogenic-oralis group of oral *Prevotella* (*Bacteroides*) spp. includes a series of non-pigmented species that share several general characteristics but give distinct results in a range of conventional biochemical tests used for bacterial identification.^{6,7,32}

The human oral strains of this group were initially thought to be similar to *B. ruminicola*, a species from the bovine rumen described by Bryant *et al.*³ in 1958, but they were later shown to be distinct. Loesche *et al.*²⁴ first used the specific name *B. oralis* for non-pigmented *Bacteroides* strains and although the original isolate was later shown to produce pigment and is now known as *P. (B.) loescheii*,^{14,19} it was subsequently shown that there was a group of gram-negative anaerobic bacilli and cocco-bacilli some of which produced pigment and others that did not. They are part of the normal flora of the gingival crevice in man and are associated with periodontal diseases and other oral infection and

with infection of soft tissue such as lung and brain abscesses.^{1,2,6,8,11} Similar organisms are also found in the vagina.^{18,25} The pathogenic role of these species is still obscure.

The taxonomy of the non-pigmented *Bacteroides* spp. has changed considerably during the last few years.^{31,32} Until recently, *B. ruminicola*,³ *B. oralis*,²⁴ *B. bivius* and *B. disiens*¹⁸ were recognised as the only non-pigmented members of the melaninogenic-oralis group of *Bacteroides*. However, some of those organisms which previously had been designated as subspecies or types of '*B. oralis*' or '*B. ruminicola*' have been elevated to species level and several new species have been described: *B. buccae* and *B. oris*,²⁰ *B. pentosaceus*,³⁰ *B. capillus*,²³ *B. zoogloiformans*,⁵ *B. heparinolyticus*,²⁶ *B. veroralis*,³⁶ *B. buccalis*³⁰ and *B. oulorum*.³³ Two species which might otherwise have been transferred to the genus *Prevotella*, *B. capillus* and *B. pentosaceus*, were amalgamated with *P. buccae* after DNA-hybridisation suggested close similarity in strains of these species.²¹ It has been proposed that the genus *Bacteroides* should be restricted to the fragilis group of *Bacteroides*, i.e.

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B. fragilis and closely related organisms.³¹ The melaninogenic-oralis group previously classified in the genus *Bacteroides* was proposed as a new genus with the name *Prevotella*.³²

Attention has been focused on the pigmented *Bacteroides* spp. and the identification of non-pigmented *Bacteroides* spp. is unsatisfactory; therefore, a reliable identification scheme is needed. The present study was designed to develop a scheme for the identification of non-pigmented *Prevotella* (*Bacteroides*) spp. by a range of conventional bacteriological tests and to apply this to strains isolated from 21 adult patients with periodontal diseases.

MATERIALS AND METHODS

Test strains

Results obtained with 13 reference strains of 12 species formed the basis of the scheme: *P. oralis* NCTC 11459, *P. veroralis* ATCC 33779, *P. buccalis* ATCC 35310, *P. oulora* NTCC 11871, *P. bivia* VPI 5540, *P. disiens* NCTC 11157, *P. buccae* ATCC 33574, *B. (P.) capillus* ATCC 33690, *P. oris* ATCC 33573, *P. zooglyphiformans* ATCC 33285, *P. heparinolytica* ATCC 35895, *B. (P.) pentosaceus* NP 333 and WPH 61. A further 91 clinical isolates from 21 adult patients with periodontal diseases were examined. The selected isolates were checked for purity by growth on BM-KV agar (BM agar, Holbrook *et al.*¹⁵ made selective for *Bacteroides* spp. by the incorporation of kanamycin 75 mg/l and vancomycin 2.5 mg/l) for 48–72 h in an anaerobic cabinet (Mark 1; Don Whitley Scientific, Shipley, W. Yorks, UK) with an atmosphere of N₂ 80 per cent, CO₂ 10 per cent, H₂ 10 per cent. Strains were harvested from purity plates and suspended in Nutrient Broth No. 2 (Oxoid) with inactivated horse serum (Wellcome) 10 per cent and glucose 1 per cent and were held at –70°C in the vapour phase of a liquid nitrogen container prior to identification.

Characterisation tests

Three approaches were used: (1) a combined set of morphology, antibiotic disk susceptibility, tolerance, biochemical and fermentation tests (conventional tests); (2) determination of volatile and non-volatile fatty acid end-products of metabolism by gas-liquid chromatography (GLC); (3) determination of a preformed enzyme profile.

(1) *Combined set of conventional tests.* Cell and colonial morphology were recorded after anaerobic incubation on BM-lysed blood agar for 48–72 h.¹⁵ Antibiotic disk susceptibility tests^{7,9} were performed with disks containing neomycin 1000 µg, kanamycin 1000 µg, penicillin 2 units and rifampicin 15 µg on BM agar. Plates were examined after anaerobic incubation for 48–72 h; strains with zones of inhibition <15 mm diameter were recorded as resistant in terms of these tests. In tolerance tests, the strains were examined for growth in the presence of sodium taurocholate, Victoria blue 4R and gentian violet by the method of Duerden *et al.*⁹

Examination for colony fluorescence was modified from the method of Slots and Reynolds,³⁵ the BM agar was flooded with 1 ml of glucose solution 1 per cent w/v, dried before inoculation, and single colonies of each strain were examined for fluorescence under u.v. light at 336 nm (Black-Ray Lamp, model UVL-21) after 1, 2, 3 and 7 d.

Biochemical tests. Strains were tested for production of indole, hydrolysis of aesculin and starch, and production of acid from glucose, lactose, sucrose, rhamnose, trehalose, xylose, maltose, arabinose, cellobiose, salicin, inulin and xylan by the methods of Duerden *et al.*^{7,9} as modified for rapid tests by Rotimi *et al.*²⁸ for hydrolysis of indoxyl acetate by the method of Popovic-Uroic *et al.*²⁷ and for hydrolysis of hippurate with Rosco Diagnostic tablets.⁴

(2) *GLC analysis.* Production of volatile (VFA) and non-volatile (NVFA) fatty acid end-products of metabolism was determined by a modification of the method of Holdeman *et al.*¹⁶ The strains were grown in Fastidious Anaerobe Broth (FAB; LabM) for 4–5 d. The FAB was supplemented with lysed horse blood 1 per cent v/v for propionic-acid negative strains (see Results). For VFA analysis, samples were acidified with 0.1 ml of H₂SO₄ 50 per cent and extracted with 0.5 ml of ether; for NVFA, cultures were treated with H₂SO₄ 50 per cent and 0.5 ml of methanol, and then incubated at 56°C for 30 min before 0.5 ml of distilled water and chloroform were added. 1 µl of sample extract was injected into a Pye Unicam PU 4550 gas chromatograph fitted with glass columns packed with FFAP (Pye Unicam), and flame-ionisation detectors; working conditions were injector 200°C, columns 180°C (VFA) or 130°C (NVFA), detector 210°C, N₂ flow rate 30 ml/min. Fatty-acid standard solutions and negative control (FAB without organisms) were included with each batch as controls.

(3) *Enzyme activities.* Strains were tested for the following enzyme activities: β -xylosidase (β -XYL), β -glucuronidase (PGUA), α - and β -glucosidase (α -GLU and β -GLU), α - and β -galactosidase (α -GAL and ONPG), α -fucosidase (α -FUC), β -n-acetylglucosaminidase (NAG), and α -mannosidase (α -MAN), with Rosco Diagnostic tablets.⁴ One tablet was added to 0.25 ml of a dense suspension of growth harvested from 48–72 h culture on BM agar in sterile saline and incubated at 35–37°C for 4 h (overnight for α -MAN). A yellow colour in the supernate denoted a positive result (strong yellow colour for NAG). In every batch of tests, one tablet was placed in 0.25 ml of uninoculated sterile saline as a negative control.

RESULTS

Reference strains

(1) *Combined set of conventional tests.* The results obtained for reference strains are shown in Table 1. The test strains were indistinguishable by microscopy of Gram-stained films, except *P. oulora* which was predominantly short rods. The other species were pleomorphic, small cocco-bacilli in pairs or short chains with rounded ends; longer rods were seen only occasionally. After incubation for 2–3 d on BM agar, colonies of most strains were circular, convex, translucent, smooth and shiny, but some strains (particularly those shown subsequently to be pentose sugar fermenting species) formed semi-opaque, cream-to-light buff colonies. Surface colonies of pentose sugar fermenting species appeared to be larger than pentose non-fermenting species after growth for 3–4 d on BM agar. The results of antibiotic disk and tolerance tests helped to distinguish this group from other *Bacteroides* spp. (e.g. the fragilis group) but did not distinguish between species within the group. Species were differentiated by results of biochemical and fermentation tests; hydrolysis of hippurate, aesculin and starch; fermentation of lactose, sucrose, rhamnose, arabinose, xylose, cellobiose, salicin, inulin and xylan; and fluorescence (Table 1). Except for *P. heparinolytica*, none of the strains produced indole, but all hydrolysed indoxyl acetate; most hydrolysed aesculin or starch, but only *P. buccalis*, *P. bivia* and *P. disiens* hydrolysed hippurate. Colonies of *P. bivia*, *P. disiens* and *P. veroralis* fluoresced under u.v. light (particularly after growth for 2–3 d). Two subgroups were separated on the basis of production of acid from pentose sugars and the species

within these subgroups were separated by other biochemical test results (Table 1).

(2) *GLC analysis.* Production of propionic, iso-valeric, iso-butyric and phenylacetic acids gave useful differentiation between species; ten patterns (i–x) were obtained with 13 reference strains (Table 2). Most species produced succinic and acetic acids as major products, and lactic acid as a minor product. Production of iso-valeric, iso-butyric, propionic and phenylacetic acids was variable. Some species produced only trace amounts of butyric or pyruvic acids. Two subgroups were distinguished by production (or not) of propionic acid (Table 2).

(3) *Enzyme activities.* Discriminatory profiles were obtained with tests for β -XYL, PGUA, β -GLU, α -GAL, α -FUC, NAG and α -MAN. Results with these enzymes gave good differentiation between species; nine patterns (a–i) were obtained with the 13 reference strains (Table 3).

Clinical isolates

On the basis of the results with reference strains, each of the three analytical approaches was found to contribute to the identification of species within the group. Therefore, all three approaches were applied to the discrimination of clinical isolates. Thirteen patterns (1–13) were obtained with the combined set of antibiotic, tolerance, biochemical and fermentation tests (Table 4), eight patterns (i–viii) were obtained with GLC analysis (Table 5) and ten patterns (a–j) for tests with enzyme activities (Table 6). There was close correlation between the three approaches and most clinical isolates could be allocated to one of ten groups (A–J) (Table 7). The commonest species were *P. buccae* (group A; 42 isolates), *P. veroralis* (group F; 15 isolates) and *P. oralis* (group E; eight isolates).

The 42 isolates of group A (with only minor variations) were identified as *P. buccae*, and most showed closely similar patterns of results; only two isolates gave identical GLC patterns to groups G and J, and one isolate gave a distinct enzyme pattern (Tables 5, 6). Six isolates (groups B and C) were biochemically similar to *P. zoogloiformans*, but four of these (group C) differed in GLC patterns and enzyme activities (Tables 5, 6). Seven isolates (group D) were identified as *P. oris*; they were distinct in conventional tests and GLC analysis, but their results in enzyme tests were variable (Table 6), and one isolate of this group gave a unique pattern in

Table 1. Patterns of results obtained with the reference strains

Test	Patterns of results*										
	1	2	3	4	5	6	7	8	9	10	11
Antibiotic-disk susceptibility											
Neomycin (1000 µg)			S						S†		
Kanamycin (1000 µg)			S						R		
Penicillin (2 units)			S/R						S/R		
Rifampicin (15 µg)			S						S		
Tolerance tests											
Sodium taurocholate	I	I	I	I	I	I	I	I	I	I	I
Victoria blue 4R	+	I	+	v	I	+	+	+	I	+	+
Gentian violet	I	I	I	I	I	I	I	I	I	I	I
Biochemical tests											
Indole production	-	-	-	+	-	-	-	-	-	-	-
Aesculin hydrolysis	+	+	+	+	+	+	+	+	-	-	-
Hippurate hydrolysis	-	-	-	-	-	-	-	+	-	+	+
Starch hydrolysis	+	v	+	+	+	w	v	-	-	+	w
Indoxyl acetate hydrolysis	+	+	+	+	+	w	w	v	+	+	+
Fluorescence	-	-	-	-	-	-	+	-	-	+	+
Pigmentation	-	-	-	-	-	-	-	-	-	-	-
Acid produced from											
Glucose	+	+	+	+	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	+	+	+	+	+	-
Sucrose	+	+	+	+	+	+	+	+	+	-	-
Rhamnose	+/-	-	-	-	-	-	-	-	-	-	-
Trehalose	-	-	-	-	-	-	-	-	-	-	-
Xylose	+	+	+	+	+	-	-	-	-	-	-
Maltose	+	+	+	+	+	+	+	+	+	+	+
Arabinose	+	+	+	+	+	-	-	-	-	-	-
Cellobiose	+	+	+	+	+	+	+	+	-	-	-
Salicin	+	+	+	+	+	+	-	-	-	-	-
Inulin	+	+	+	v	-	w	+	v	-	-	-
Xylan	+	-	-	-	-	-	w	-	-	-	-

*Pentose +ve species: 1—*P. buccae* ATCC 33574, *B. (P) capillus* ATCC 33690; 2—*P. zooglyphiformans* ATCC 33285; 3—*B. (P) pentosaceus* NP333 and WPH61; 4—*P. heparinolytica* ATCC 35895; 5—*P. oris* ATCC 33573. Pentose -ve species: 6—*P. oralis* NCTC 11459; 7—*P. veroralis* ATCC 33779; 8—*P. buccalis* ATCC 35310; 9—*P. oulora* NCTC 11871; 10—*P. bivia* VPI 5540; 11—*P. disiens* NCTC 11157.

Results: S = sensitive; R = resistance; S/R = most strains were sensitive; in tolerance tests; I = inhibition; + = growth; hydrolysis or acid production in biochemical tests; + = positive reaction; - = negative reaction; +/- = most reactions positive; -/+ = most reactions negative; w = weak reaction; v = variable reaction; p = pale fluorescence; r = red fluorescence.

†*P. oulora* was resistant.

enzyme tests and one isolate was similar to group B. Eight isolates with only minor variations in biochemical and fermentation tests were identified as *P. oralis* (group E) and almost gave unique patterns of results in all three approaches, except four isolates that gave enzyme patterns identical to groups F and G (Tables 4–6). The 15 isolates in group F formed an heterogeneous group in conventional tests, but gave almost identical patterns in GLC and enzyme tests.

These were closely similar to the reference strain of *P. veroralis*, but differed in producing trace amount of iso-valeric acid and red fluorescence under u.v. light. One isolate (group G) was slightly similar to *P. buccalis*, but gave a pattern in enzyme activities identical to group F, and a fatty acid pattern identical to group J (Tables 5, 6). One isolate (group H) gave unique patterns of results in all three approaches and was fairly close to *P. oulora*. Four

Table 2. Patterns of volatile and non-volatile fatty acid (VFA and NVFA) production by reference strains

Fatty acid	Amount of acid produced*									
	i	ii	iii	iv	v	vi	vii	viii	ix	x
VFA										
Acetic	++	+	+	++	++	++	++	++	++	++
Propionic	tr	++	++	+	++	-	-	-	-	-
iso-Butyric	tr	++	+	-	-	-	tr	+	++	tr
Butyric	-	-	-	-	tr	tr	-	-	tr	tr
iso-Valeric	++	++	++	+	tr	tr	++	++	++	+
NVFA										
Pyruvic	-	-	-	-	-	-	tr/-	tr	-	-
Lactic	tr	tr	tr	tr	+	+	+	+	+	+
Succinic	++	++	++	++	++	++	++	++	++	++
Phenylacetic	-	+	+	-	-	tr	-	-	-	-

*Propionic +ve species: i—*P. buccae* ATCC 33574, *B. (P) capillus* ATCC 33690; ii—*P. zoogloformans* ATCC 33285, *B. (P) pentosaceus* NP333 and WPH61; iii—*P. heparinolytica* ATCC 35895; iv—*P. oris* ATCC 33573; v—*P. oulora* NCTC 11871. Propionic -ve species: vi—*P. oralis* NCTC 11459; vii—*P. veroralis* ATCC 33779; viii—*P. buccalis* ATCC 35310; ix—*P. bivia* VPI 5540; x—*P. disiens* NCTC 11157.
Results: ++ = major production; + = minor; tr = trace; - = none produced.

Table 3. Enzyme activities of the references strains

Enzyme*	Pattern of results†								
	a	b	c	d	e	f	g	h	i
β-XYL	+	-	-	+	w	-	-	-	-
PGUA	-	+	-	+	-	-	-	-	-
α-GLU	+	+	+	+	+	+	+	+	+
β-GLU	+	+	+	+	+	+	-	-	-
α-GAL	+	+	+	+	+	+	+	-	+
ONPG	+	+	+	+	+	+	+	v	-
α-FUC	-	+	+	+	+	+	-	+	-
NAG	-	+	+	+	+	+	w	w	-
α-MAN	-	v	-	+	-	v	+	+	v

*β-XYL = β-xylosidase; PGUA = β-glucuronidase; α-GLU = α-glucosidase; β-GLU = β-glucosidase; α-GAL = α-galactosidase; ONPG = β-galactosidase; α-FUC = α-fucosidase; NAG = β-n-acetylglucosaminidase; α-MAN = α-mannosidase.

†a—*P. buccae* ATCC 33574, *P. (P) capillus* ATCC 33690; b—*P. zoogloformans* ATCC 33285, *P. oris* ATCC 33573; c—*B. (P) pentosaceus* NP333 and WPH61; d—*P. heparinolytica* ATCC 35894; e—*P. oralis* NCTC 11459; f—*P. veroralis* ATCC 33779, *P. buccalis* ATCC 35310; g—*P. oulora* NCTC 11871; h—*P. bivia* VPI 5540; i—*P. disiens* NCTC 11157.

Results: + = positive reaction; - = negative reaction; +/- = most reactions positive; v = variable reactions; w = weak reaction.

Table 4. Patterns of results obtained with the clinical isolates in conventional tests

Test	Patterns of results*												
	Pentose +ve strains				Pentose -ve strains								
	1	2	3	4	5	6	7	8	9	10	11	12	13
Antibiotic-disk susceptibility													
Neomycin (1000 µg)			S				S		S				
Kanamycin (1000 µg)			R				R		R				
Penicillin (2 units)			S/R				S/R		S				
Rifampicin (15 µg)			S				S		S				
Tolerance tests													
Sodium taurocholate	I	I	I		I	I	I	I	I	I	I	I	I
Victoria blue 4R	+	I	+/I		+	I	+	v	+	I	+	I	v
Gentian violet	I	I	I		I	I	I	I	I	I	I	I	I
Biochemical tests													
Indole production	-	-	-	-	-	-	-	-	-	-	-	-	-
Aesculin hydrolysis	+	+	+	+	+	+	+	+	+	w	-	-	v
Hippurate hydrolysis	-	-	-	-	-	-	-	v	+	-	+	-	+
Starch hydrolysis	+	v	+	-	+	+	-	+	w	-	+	-	v
Indoxyl acetate	+	+	+	+	+	v	+	w	v	+	+	w	+
Fluorescence	-	-	+ ^p	-	-	+ ^r	+ ^r	v	-	-	+ ^r	+ ^r	+ ^r
Pigmentation	-	-	-	-	-	v	v	v	-	-	-	-	-
Acid produced from													
Glucose	+	+	+	+	+	+	+	+	+	+	+	w	+
Lactose	+	+	+	+	+	+	+	+	+	+	+	w	+
Sucrose	+	+	+	+	+	+	+	+	+	+	-	w	+
Rhamnose	+/-	-	-	-	-	-	-	-	-	-	-	-	-
Trehalose	-	-	-	-	-	-	-	-	-	-	-	-	-
Xylose	+	+	+	+	-	-	-	-	-	-	-	-	-
Maltose	+	+	+	+	+	+	+	+	+	+	+	+	+
Arabinose	+	+	+	+	-	-	-	-	-	-	-	-	-
Cellobiose	+	+	+	+	+	+	+	-	-	-	-	-	-
Salicin	+	+	+	+	+/-	-	-	-	-	-	-	-	-
Inulin	+/-	+	-	-	w	-	+	-	w	-	-	-	-
Xylan	+/-	-	-	-	-	-	+	w	-	-	-	-	-
No. of isolates	42	6	5	2	8	6	6	3	1	1	4	5	2
Groups (overall)	A	B,C	D		E		F		G	H	I	J	

*See footnote to Table 1. S/R = some strains gave each result, more strains were sensitive; +/I = more strains were grown; + = >95 per cent of strains gave a positive result; - = >95 per cent of strains gave a negative result.

isolates (group I) gave very similar patterns of results in conventional tests and GLC analysis and corresponded with *P. bivia*, but gave identical patterns in enzyme activities with group J (Table 6). The strains of group I differed from *P. bivia* by production of α -GAL. Seven isolates (group J) gave two distinct patterns in conventional tests and did not correspond with any reference strains, but gave

GLC patterns identical with group G and enzyme activities identical with group I (Tables 5, 6).

DISCUSSION

Non-pigmented *Bacteroides* of the melanogenicus-oralis group (*Prevotella* spp.) have received less attention than pigmented species, but may be

Table 5. Patterns of VFA and NVFA production by clinical isolates

	Amount of acid produced*							
	Propionic + ve strains				Propionic - ve strains			
	i	ii	iii	iv	v	vi	vii	viii
VFA								
Acetic	++	+	++	++	++	++	++	++
Propionic	tr	++	+	++	-	-	-	-
iso-Butyric	tr	++	-	-	-	tr	++	+/tr
Butyric	-	-	tr	tr	tr	-	-	-
iso-Valeric	++	++	tr	+	tr	+/-	++	++
NVF								
Pyruvic	-	-	-	+	-	tr/-	-	tr/-
Lactic	tr	tr	tr	+	+	+	+	+
Succinic	++	++	++	++	++	++	++	++
Phenylacetic	-	+	-	-	tr	-	-	-
No. of isolates	40	2	7	1	8	14	4,4	2,1,1,7
Groups (overall)	A	B	D	H	E	F	C,I	A,F,G,J

*See footnote to Table 2.

Table 6. Enzyme activities of the clinical isolates

Enzyme	Patterns of results*									
	a	b	c	d	e	f	g	h	i	j
β -XYL	+	+	+	+	-	-	+	-	-	-
PGUA	-	+	+	-	+	-	-	-	-	-
α -GLU	+	+	+	+	+	+	+	+	+	+
β -GLU	+	+	+	+	+	+	+	+	-	-
α -GAL	+	+	+	+	+	+	+	+	-	+
ONPG	+	+	+	+	+	-	+	+	-	+/ -
α -FUC	-	-	+	+	+	-	+	+	+	+
NAG	-	-	+	+	+	+	+	+	+	+
α -MAN	-	-	+	+	v	-	-/+	+/-	w	+/-
No. of isolates	41	1	2,1	4,1	5	1	4	4,15,1	1	4,6
Groups (overall)	A	A	B,D	C,J	D	D	E	E,F,G	H	I,J

*See footnote to Table 3.

significant pathogens. Until now, little information has been available about the characteristics and prevalence of non-pigmented *Prevotella* spp. in infections of the oral cavity.¹¹ In the past few years, several new species^{5,20,23,26,30,33,36} have been described but few attempts have been made to

characterise clinical isolates in view of recent taxonomic changes.

P. buccae and *P. oris* are the most commonly isolated non-pigmented *Prevotella* spp. and are found mostly in the oral cavity.^{12,22} They are phenotypically identical to the animal isolate *B. ruminicola*

Table 7. Results (overall) obtained of clinical isolates compared with reference strains of prevotella

Species (reference)	Nos. (clinical)	Groups	Good fit	Minor variation	Not identified
<i>P. buccae</i>	42	A	41	1	0
<i>P. zoogloformans</i>	6	B,C	2	4	0
<i>P. oris</i>	7	D	3	4	0
<i>P. oralis</i>	8	E	7	1	0
<i>P. veroralis</i>	15	F	6	9	0
<i>P. buccalis</i>	1	G	0	1	0
<i>P. oulora</i>	1	H	0	1	0
<i>P. bivia</i>	4	I	4	0	0
Undesignated	7	J	0	0	7
Total	91		63	21	7

subsp. *brevis*, from which they have been separated as new species by DNA homology and SDS-PAGE studies.²⁰ Although *P. buccae* and *P. oris* are indistinguishable by many conventional biochemical tests,²⁰ the results of this study show that they may be differentiated by GLC analysis and tests for enzyme activity (Tables 2, 3). We also found that *P. buccae* (group A) was more common than *P. oris* (group D; Table 7) amongst isolates from adult periodontal disease and this finding indicates that *P. buccae* may have an important role in the human mouth.

B. (P.) pentosaceus NP 333 and WPH 61 and *B. (P.) capillus* ATCC 33690 have been shown to be phenotypically similar to *P. buccae* ATCC 33574. Johnson and Holdeman²¹ found that *P. buccae* ATCC 33574, *B. (P.) capillus* ATCC 33690 and *B. (P.) pentosaceus* NP 333 have more than 80 per cent DNA homology and suggested that the three names were synonymous. Since *P. buccae* Holdeman *et al.*²⁰ was the first validly published name for this taxon, *B. (P.) capillus* and *B. (P.) pentosaceus* would be regarded as later synonyms. Haapasalo *et al.*¹³ also demonstrated homogeneity among the ultra-structure of the S-layer of *B. (P.) capillus* ATCC 33690, *B. (P.) pentosaceus* NP 333 and WPH 61 and *P. buccae* ATCC 33574 by electron microscopy and suggested that isolates with an S-layer may belong to the same species. Our results showed that *P. buccae* ATCC 33574 and *B. (P.) capillus* (ATCC 33690) had overlapping characters in all three approaches. However, *B. (P.) pentosaceus* NP 333 and WPH 61 differed from *P. buccae* and *B. (P.) capillus* in failing to ferment xylan, and in producing

propionic (major) and phenylacetic (minor) acids, α -FUC and NAG (Tables 1-3). *B. (P.) pentosaceus* NP 333 and WPH 61 differed from *P. zoogloformans* 33285 only in tolerance of Victoria blue 4R, failure to produce PGUA and variable starch hydrolysis (Tables 1, 3) and were therefore more similar to *P. zoogloformans* than to *P. buccae*.

Limited work has been done on the characterisation of *P. zoogloformans*¹¹ (*Capsularis zoogloformans* Weinberg *et al.*,³⁷ emended later to *Bacteroides zoogloformans* Cato *et al.*⁵). The most distinctive property of this species is growth as a viscous, glutinous mass that is difficult to remove from agar media.⁵ In this study the reference strain of *P. zoogloformans* produced propionic (major) and phenylacetic (minor) acids. However, of the six clinical isolates (groups B, C) which were similar to *P. zoogloformans* ATCC 33285 in showing typical viscosity, four strains (group C) did not produce propionic acid or phenylacetic acids and were β -XYL positive (Tables 5, 6). *P. heparinolytica* ATCC 35895 gave patterns in GLC and conventional tests very similar to those of *P. zoogloformans* and produced similar viscosity. *P. heparinolytica* was distinguished from *P. zoogloformans* by production of indole and β -XYL, and growth on Victoria blue 4R. The viscosity of *P. zoogloformans* and *P. heparinolytica* may be a significant factor for attachment of the organism to the surfaces of teeth or to other parts of the oral cavity. However, this property and its relationship to other characteristics need to be studied further.

The taxonomic status of the species *P. oralis* has long been controversial. The identification of the 16

strains originally isolated by Loesche *et al.*,²⁴ was thought to be doubtful by Shah and Collins.³⁰ In view of the unsatisfactory status of the species, Shah and Collins²⁹ suggested that it should contain only those strains (typified by strains VPI 8906D, HS4) conforming closely to the original description of *P. oralis*. However, in the Approved Lists of Bacteriological Names,³⁴ strain ATCC 33269 was designated the new type strain of *P. oralis*;³⁰ strains VPI 8906D and HS4 were assigned to the new species *P. buccalis*³⁰ and strain VPI D22A-7 to *P. veroralis*.³⁶

In conventional biochemical tests *P. oralis*, *P. veroralis* and *P. buccalis* are similar, except that *P. oralis* ferments salicin and *P. buccalis* does not hydrolyse starch. We found that: *P. oralis* was distinguished by GLC and enzyme tests (Tables 2, 3), but *P. veroralis* and *P. buccalis* gave very similar patterns in both; *P. buccalis* was differentiated from *P. veroralis* only by hydrolysis of hippurate (Table 1). In the original description³⁰ of *P. buccalis* it was shown as not hydrolysing starch and our results confirm this finding. However, *P. buccalis* strains studied later by Holdeman *et al.*¹⁷ hydrolysed starch. In the present study eight isolates (group E) showed biochemical and GLC patterns identical to the reference strain of *P. oralis*, but four of these differed in their enzyme profiles, which were similar to *P. veroralis* (Tables 4–6). The 15 clinical isolates (group F) that corresponded with *P. veroralis* showed some variation in biochemical and fermentation tests, but they gave similar patterns in enzyme tests, all fluoresced under u.v. light and most produced a small amount of dark pigment in the centre of the colonies after incubation for 6 d (Tables 4–6).

P. oulora,³³ previously named '*B. oralis*',^{10,16} does not ferment pentose sugars. Data on other characters and on the occurrence of *P. oulora* in human infection are also limited; the reference strain was distinct from other species in all three approaches and only one clinical isolate was slightly similar to this species (Tables 4–6).

P. bivia and *P. disiens* were usually isolated from the female genital tract,^{18,25} and were originally described as *B. melaninogenicus*, but did not form pigmented colonies. However, they have a low level of DNA homology with *B. melaninogenicus* subsp. *intermedius*.¹⁸ They are distinguished readily from other non-pigmented *Prevotella* spp. by negative results in tests for aesculin hydrolysis and production of red fluorescence under u.v. illumination, and their hydrolysis of hippurate and characteristic pattern of sugar fermentation (Table 1). Although the

two species differ only in lactose fermentation in conventional tests, they were clearly discriminated in enzyme tests. The four clinical isolates of group I gave results corresponding with *P. bivia*, but no clinical isolates corresponded with *P. disiens* (Tables 4–7).

Of the seven clinical isolates that could not be identified, five showed distinctive growth characteristics (slow and fastidious growth), weak fermentation of sugars, and fluoresced under u.v. light (Tables 4–7). These five strains appeared to be a distinct and homogeneous group worthy of species status.

Our results indicate that oral non-pigmented *Prevotella* spp. can be differentiated from the other groups of gram-negative anaerobic bacilli by resistance to kanamycin and sensitivity to rifampicin and neomycin, inhibition by bile salt and gentian violet, and production of a major amount of acetic and succinic acids, and minor amount of iso-valeric and lactic acids. They can be separated into two subgroups: A, those that ferment pentose sugars, produce propionic acid and grow much more rapidly and to a higher turbidity (*P. heparinolytica*, *P. zoogloformans*, *B. (P.) pentosaceus*, *P. buccae*, *P. oralis*, and *B. (P.) capillus*); and subgroup B (*P. oralis*, *P. veroralis*, *P. buccalis*, *P. oulora*, *P. bivia* and *P. disiens*) that do not share these properties (although *P. oulora* produced propionate and *P. bivia* gave heavy growth).

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