A comparison of *Bacteroides ureolyticus* isolates from different clinical sources

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Summary. Clinical isolates of corroding, gram-negative, anaerobic bacilli (provisionally identified as *Bacteroides ureolyticus*) from superficial ulcers and soft tissue infections (15), non-gonococcal, non-chlamydial urethritis (12) and adult periodontal disease (14) were compared with reference strains of *B. ureolyticus*, *B. gracilis* and *Wolinella recta* in a series of conventional tests of morphology, biochemical activity, tolerance of dyes and bile salts, and antibiotic sensitivity, gas-liquid chromatographic analysis of metabolic products, and in whole-cell analysis by pyrolysis-mass spectrometry (Py-MS). A numerical taxonomic approach was used with the results of conventional tests and the grouping obtained was compared with that obtained by Py-MS. All the ulcer and soft-tissue isolates and the urethritis isolates were oxidase- and urease-positive and formed a homogeneous set consistent with the reference strain of *B. ureolyticus*. The dental isolates differed from *B. ureolyticus* strains and were heterogeneous amongst themselves. None corresponded with the reference strains of *B. gracilis* or *W. recta*. The conventional and Py-MS approaches to characterisation produced similar groupings and each distinguished between a single cluster of ulcer-urethritis strains and several clusters of dental strains, although the dendrograms derived from the two approaches differed in the order of the clusters; in the Py-MS dendrogram one subcluster of four dental strains came within the main ulcer-urethritis cluster and a cluster of five ulcer strains was separated as a distinct group.

Introduction

Clinical anaerobic isolates of gram-negative bacilli that produce pitting or corroding growth on agar-based media were first described by Henriksen (1948). Eiken (1958) isolated similar organisms from abscesses, mostly of the buccal region, and named them *Bacteroides corrodens*. Subsequently there were sporadic reports of *B. corrodens* from abscesses (Reinhold, 1966), gingival crevice samples and blood cultures after tooth extraction (Khairat, 1967), and diseased tonsils (Schroter and Stawru, 1970). These early reports are difficult to interpret because no distinction was drawn between those corroding strains that were strict anaerobes and those that were CO₂-dependent and facultatively anaerobic. Jackson and Goodman (1972) made this distinction, allocating the facultative strains to the sole species of a new genus, *Eikenella*

(*E. corrodens*), and reserving the name *B. corrodens* for the strict anaerobes. Subsequently they proposed that corroding *Bacteroides* strains should be re-named *B. ureolyticus* to avoid confusion with *E. corrodens* and to reflect another distinctive property of *B. ureolyticus*—strong urease activity (Jackson and Goodman, 1978).

Although this re-classification confirmed the separation of *E. corrodens* and *B. ureolyticus*, it did not resolve the taxonomic problem completely and the classification of *B. ureolyticus* in the genus *Bacteroides* remains in doubt. It differs from other *Bacteroides* spp. in the low G+C content of its DNA—28–30 mol %, compared to 40–52 mol % for most “true” *Bacteroides* spp. (Shah and Collins, 1983)—and in giving positive results in oxidase and urease tests. *B. ureolyticus* does not produce acid from carbohydrates, but, unlike the *Fusobacterium* spp. which are also asaccharolytic, it does not produce *n*-butyric acid. Duerden (1983) grouped *B. ureolyticus* with other asaccharolytic *Bacteroides*

spp. but it is doubtful whether any asaccharolytic species should remain within the genus *Bacteroides* and it has been proposed that the pigmented asaccharolytic species, *B. asaccharolyticus*, *B. gingivalis* and *B. endodontalis*, should be transferred to a new genus, *Porphyromonas* (Shah and Collins, 1988). Studies of 16S ribosomal ribonucleic acid sequences suggest that *B. ureolyticus*, two similar species *B. gracilis* and *Wolinella recta*, and *Campylobacter* spp. form a single taxon (Paster and Dewhirst, 1988). *B. gracilis* is an anaerobic, corroding species, similar to *B. ureolyticus* in cultural and morphological characters, but it is oxidase-negative, does not produce urease, and has a DNA G + C content of 44–46 mol %. *W. recta* and *B. gracilis* are indistinguishable in most biochemical tests but differ in that *W. recta* is motile and has a curved cell morphology (Tanner *et al.*, 1981).

The sporadic reports of the isolation of *B. ureolyticus* from clinical specimens have generally emphasised its association with certain pathogenic processes. These include: buccal abscesses (Eiken, 1958); periodontal disease (Khairat, 1967; Duerden *et al.*, 1987) and other oral infections (Schroter and Stawru, 1970; Duerden *et al.*, 1982); soft tissue infection and ulcers, especially peripheral (e.g., varicose) ulcers and gangrene, and ulcerative conditions of the perineum and external genitalia (Labbé *et al.*, 1977; Duerden *et al.*, 1982; Masfari *et al.*, 1983, 1985). Fontaine *et al.* (1982, 1984) used refined anaerobic transport and culture methods to isolate strains, subsequently confirmed as *B. ureolyticus* (Fontaine *et al.*, 1986; Taylor *et al.*, 1986) from urethral samples from men with non-gonococcal, non-chlamydial urethritis; successful treatment with metronidazole further supported a pathogenic role for *B. ureolyticus* in these patients (Hawkins *et al.*, 1988). Taylor *et al.* (1987) examined isolates from urethritis and other conditions by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of protein extracts and by conventional biochemical tests; they concluded that strains from the various sources were similar but were able to divide the species into four types on the basis of SDS-PAGE patterns.

In a series of investigations of anaerobic infections, we have isolated *B. ureolyticus* from a range of clinical conditions in which it seemed to have a pathogenic role (Duerden *et al.*, 1982, 1987; Masfari *et al.*, 1983, 1985). In the present study, we examined three groups of isolates—from dental sources, from ulcers and soft-tissue infections (including genital ulcers), and from urethritis—in morphological, biochemical, tolerance and antibiotic susceptibility tests, by GLC analysis of volatile fatty-acid prod-

ucts of metabolism, and by pyrolysis-mass spectrometry (Py-MS), and compared these with reference strains of *B. ureolyticus* and *B. gracilis*.

Materials and methods

B. ureolyticus and other reference strains

Three groups of isolates were examined: 15 isolates from soft tissue infections and ulcers, including perineal, genital and peripheral ulcers; 14 dental isolates from patients with adult periodontal disease; and 12 isolates from men with non-gonococcal, non-chlamydial urethritis. The ulcer and dental strains were isolated in our laboratories; the urethritis strains were provided by Dr E. A. R. Fontaine, Division of Sexually Transmitted Diseases, Clinical Research Centre, Harrow. Four reference strains were also examined: *B. ureolyticus* NCTC 10941, *B. gracilis* ATCC 33236, *B. gracilis* WAL 85/253C (provided by Dr S. Finegold, Wadsworth Anaerobe Laboratory, Los Angeles, CA) and, in some tests, *W. recta* NCTC 11489.

All strains were stored in the vapour phase of a liquid nitrogen container as suspensions of growth from blood agar (48–72 h, anaerobic incubation, 36.5°C) in glucose 10% and horse serum 10% in BM broth (Williams *et al.*, 1975).

Morphology

Colony appearance was recorded after incubation for 48 and 96 h on Columbia Agar (Oxoid) with defibrinated horse blood (Oxoid) 7.5%, and on BM-lysed blood agar (Holbrook *et al.*, 1978) with particular reference to pitting or spreading growth. All incubation was at 36.5°C in an anaerobic cabinet (Mark 1; Don Whitley Scientific, Shipley, W. Yorks) with an atmosphere of N₂ 80%, CO₂ 10%, H₂ 10%.

Cell morphology was examined in gram-stained films from growth on blood agar after anaerobic incubation for 48 h.

Motility. Strains were examined for evidence of motility by microscopy (at a magnification of 400 with reduced illumination) of plain wet preparations from 24- and 48-h cultures in BM broth.

Biochemical tests

Confirmation that strains were strict anaerobes was sought by incubating subcultures on blood agar in air + CO₂ 10% for 96 h.

Strains were tested for production of indole and lipase, hydrolysis of aesculin, digestion of charcoal-gelatin disks, and production of acid from glucose, lactose, sucrose, rhamnose, cellibiose, salicin, galactose and xylan by the methods of Duerden *et al.* (1976, 1980) as modified for rapid tests by Rotimi *et al.* (1980).

Oxidase test. Growth from blood agar was smeared on filter paper soaked in fresh N-tetramethyl *p*-phenylene-

diamine solution. A blue or purple colour developing within 10 s was a positive result. Control organisms were *Pseudomonas aeruginosa* (positive) and *Klebsiella aerogenes* (negative).

Urease tests were performed with Urease Diagnostic Tablets (Rosco Diagnostica; LabM, Salford, Lancs). A tablet was added to 0.25 ml of a dense saline suspension from a 48-h blood-agar culture and incubated at 37°C for 4 h. A red-purple colour denoted a positive test; negative tests remained yellow-orange. *Proteus mirabilis* was the positive control organism.

Nitrate reduction tests were performed with Nitrate Reduction Diagnostic Tablets (Rosco Diagnostica; LabM; Wideman *et al.*, 1977) that contain sodium molybdate and potassium nitrate. A tablet was added to 0.25 ml of a dense saline suspension as above. After incubation at 37°C for 4 h, one drop of dimethyl- α -naphthylamine, 0.6% in 5N acetic acid, and one drop of sulphanilic acid, 0.8% in 5N acetic acid were added. A red-pink colour developing within 2 min denoted a positive test (reduction to nitrite); negative tests remained pale pink or colourless. Zinc dust was added to all negative tubes to detect the presence of nitrate; failure to produce a red-pink colour indicated reduction beyond nitrite.

Tolerance tests

Strains were tested for their ability to grow in the presence of sodium taurocholate (Sigma, Poole, Dorset), Victoria blue 4R, gentian violet, ethyl violet, brilliant green and methyl green (BDH, Atherstone, Warwickshire) by the streak-culture method of Duerden *et al.* (1976). Sodium taurocholate and the dyes were dissolved in distilled water and incorporated in BM agar or Fastidious Anaerobe Agar (FAA; LabM) supplemented with sodium formate 1 g/L and sodium fumarate 1.5 g/L (FAAS), at final concentrations of 5 g/L for sodium taurocholate, 1 in 100 000 for gentian violet and 1 in 80 000 for the other dyes. Test strains were grown for 48 h on FAAS and then streaked on the dye-containing plates, which were examined for growth after anaerobic incubation at 36.5°C for 48 h.

Antibiotic susceptibility tests

Disk resistance tests (Duerden *et al.*, 1976, 1980) were performed with metronidazole (5 μ g), neomycin (1000 μ g), kanamycin (1000 μ g), penicillin (2 units) and rifampicin (15 μ g) disks on blood agar; the inoculum was taken directly from a 48-h culture on blood agar. Plates were examined after anaerobic incubation for 48 h; strains with zones of inhibition $\leq$ 15 mm diameter were recorded as resistant in terms of this test.

MIC determinations were made by an agar dilution method (Jones *et al.*, 1985). The antibiotics used and ranges of concentrations tested were: benzyl penicillin (Glaxo Laboratories Ltd, Greenford, Middx) 0.03–64 mg/L, vancomycin (Eli Lilly, Basingstoke) 0.25–

16 mg/L, nalidixic acid (LabM) 0.125–128 mg/L, polymyxin B (Sigma) 0.03–4.8 i.u./ml and teicoplanin (provided by Merrell Dow, Egham, Surrey) 0.03–128 mg/L. Antibiotic solutions were freshly prepared by dissolving weighed powder in sterile distilled water and serial dilutions were added to FAAS. The strains were grown anaerobically on blood agar for 48 h and a suspension was prepared in Fastidious Anaerobe Broth (FAB; LabM) to match Wellcome opacity tube no. 1 (equivalent to $c. 10^9$ cfu/ml.) and diluted 1 in 100. A multipoint inoculator (Denley Instruments, Billingham, Sussex) was used to deliver a 1- μ l inoculum containing $c. 10^4$ cfu. Plates were incubated anaerobically at 36.5°C and examined for the presence or absence of growth after 48 h.

GLC analysis

Volatile fatty-acid (VFA) end-products of metabolism were determined by gas-liquid chromatography (GLC) after growth on agar media and in broth. Difficulty was experienced in obtaining satisfactory growth in broth or reproducible results after growth on agar. The broth medium used were: FAB; FAB + fumarate, formate, succinate and pyruvate supplements; and *B. ureolyticus* medium with supplements (Fontaine *et al.*, 1982); blood was not included in the formulations. The agar media were: Columbia Blood Agar (Oxoid), *B. ureolyticus* agar with supplements (Fontaine *et al.*, 1982), and FAA + blood 7.5%. For broth cultures, 1 ml of culture was shaken with 0.5 ml of 50% H₂SO₄ and 1 ml of ether and then centrifuged. For agar cultures, an agar square $c. 3 \text{ cm} \times 3 \text{ cm}$ was macerated and added to 1 ml of 50% H₂SO₄ + 1 ml of ether; the mixture was shaken intermittently for 30 min and centrifuged. For GLC, 1 μ l of ether extract was injected into a Pye Unicam series 204 Gas Chromatograph equipped with a flame ionisation detector and two 1.5 m $\times$ 4 mm glass columns packed with CAW (80–100 mesh) coated with 10% FFAP. Running conditions were: carrier gas flow rate 30 ml/min; injector temperature 190°C; column temperature 160°C; detector temperature 200°C. Fatty-acid standard solutions (Supelco, R. B. Radley & Co. Ltd, London Road, Sawbridgeworth, Herts, CM21 9JH) were included with each batch as controls.

Pyrolysis-mass spectrometry (Py-MS)

Whole-cell composition was examined by Py-MS in a Horizon Instruments PYMS 200X pyrolysis-mass spectrometer (Aries *et al.*, 1986; Magee *et al.*, 1989). Growth from 48-h cultures on blood agar was smeared on iron-nickel alloy pyrolysis foils and heated rapidly in a vacuum to 530°C by the Curie point method. The volatile low-mol. wt pyrolysis products were ionised by low energy electron impact and separated according to mass-charge ratio in the quadrupole mass spectrometer to yield a quantitative mass spectrum. Quadruplicate analyses were performed on each strain.

Spectra were normalised by the iterative technique of

Huff *et al.* (1981) to correct for differences in sample size. The normalisation set comprised masses 51–60, 62–75, 77–89, 93–106, 108–113, 116, 118–128, 132–136, 138–140, 147, 149, 150, 153, 154 and 156. Masses showing a mean within-strain coefficient of variation $\leq 15\%$ were selected for further analysis as previously described (Magee *et al.*, 1989); these comprised masses 51–60, 62–75, 77–89, 93–106, 108–113, 116, 118–128 and 132–136. Replicate spectra of each strain were labelled as a distinct group and normalised data for the selected peaks were subjected to discriminant analysis (SPSSX User's Guide, 1983). This optimised discrimination between the strains, correcting the data for systematic covariance, and for differences in within-strain reproducibility. The spectra could now be represented by co-ordinates on 30 statistically corrected independent (orthogonal) canonical discriminant function axes, accounting for 100% of inter-strain variance. Mean strain spectrum co-ordinates on these axes were processed in classification analyses programmed in Clustan 3.2 (Wishart, 1982). Squared euclidean distance (D^2) between spectral points in the space described by the 30 discriminant functions was the index of dissimilarity for Py-MS data. This index is approximately equal to $\chi^2/30$ (29 degrees of freedom), with the null hypothesis that the pairs of spectra compared were derived from the same strain. Four clustering strategies were explored—hierarchical clustering by UPGMA (unweighted pair group arithmetic average; Sokal and Michener, 1958) and Ward's method (Ward, 1963), and non-hierarchical clustering by Relocate (Crawford and Wishart, 1968) and Normix (Wolfe *et al.*, 1970); classifications from both hierarchical methods were used as starting points for non-hierarchical clustering. Cophenetic correlations within the similarity matrix were calculated for hierarchical methods, and probabilities of N groups (p of null hypothesis) for Normix (Wolfe *et al.*, 1970). Normalisation and peak selection were performed in BASIC VM on a Prime 750 computer, and discriminant and classification analyses on an IBM 3083 computer at the Sheffield University Computer Centre.

Comparison of Py-MS and conventional grouping

A numerical taxonomic approach was used with the results of tests for oxidase and urease production, nitrate reduction, susceptibility to neomycin, kanamycin, penicillin and nalidixic acid, and tolerance of brilliant green, Victoria blue 4R, gentian violet, ethyl violet and methyl green. Simple matching (Sokal and Michener, 1958) and the Jaccard coefficient (Sneath, 1957) were the indices of similarity for the data from conventional tests; the cluster analyses used were UPGMA, Ward's method and Relocate non-hierarchical clustering. The grouping of strains obtained in the simple matching+UPGMA strategy was compared with that obtained by Py-MS.

Results

All test strains were strictly anaerobic gram-negative bacilli that were sensitive to metronidazole

and did not grow after incubation at 37°C in air + CO₂ 10% for 4 days.

Morphology

Colonies. All putative *B. ureolyticus* strains produced pitting (corroding) growth at some stage during the studies. All strains from the ulcer and urethritis groups produced pitting (corroding) growth on primary isolation and continued to do so on blood agar, but subcultures did not pit BM agar. On primary isolation, most dental strains produced feathery, spreading growth, rather than pitting, but subcultures on BM agar or blood agar produced pitting growth. The reference strains of *B. gracilis* and *W. recta* did not pit any agar medium used.

Cells. The test strains were indistinguishable by microscopy of gram-stained smears. All were pale-staining, slender, regular gram-negative bacilli.

Motility. The reference strain of *W. recta* was actively motile; many cells showed tumbling or darting motility. The reference strains of *B. gracilis* and *B. ureolyticus* were generally non-motile but occasional cells of each strain appeared to show some tumbling motility. Ten of the urethritis isolates and 12 of the ulcer isolates were non-motile; with the other isolates, occasional cells appeared to be motile. Six of the dental isolates were non-motile and eight were clearly motile (table I).

Biochemical tests

None of the test strains produced indole or lipase, hydrolysed aesculin, or produced acid from any of the carbohydrates but all digested gelatin.

The results of the oxidase, urease and nitrate reduction tests are shown in table II. Ulcer and urethritis strains gave consistent positive results in

Table I. Results of microscopy to determine motility of test strains in 48-h broth culture

Strains	n	Motility		
		+	±	–
Ulcer	15		3	12
Urethritis	12		2	10
Dental	14	8		6
<i>B. ureolyticus</i> NCTC 10941	1		1	
<i>B. gracilis</i>	2		2	
<i>W. recta</i>	1	1		

+ = Actively motile; ± = occasional cells motile;
– = non-motile.

Table II. Results of tests for oxidase and urease production and for reduction of nitrate

Strains	n	Oxidase		Urease		Nitrate reduction	
		+	-	+	-	+	-
Ulcer	15	15	0	15	0	11	4
Urethritis	12	12	0	12	0	9	3
Dental	14	5	9	7	7	10	4
<i>B. ureolyticus</i> NCTC 10941	1	1	0	1	0	1	0
<i>B. gracilis</i>	2	0	2	0	2	1	1
<i>W. recta</i>	1	0	1	0	1	1	0

oxidase and urease tests but the dental strains gave mixed results. A majority of strains in each group reduced nitrate.

Tolerance tests

All strains were inhibited by sodium taurocholate but grew on the media containing brilliant green, methyl green or Victoria blue 4R. All the ulcer and urethritis isolates and the reference strains of *B. ureolyticus* and *B. gracilis*, were tolerant of ethyl violet but half of the dental strains were inhibited.

The results with gentian violet were varied, the reference strains of *B. gracilis*, three ulcer and three urethritis strains and two dental strains were tolerant. All the dental strains that were inhibited by ethyl violet were also inhibited by gentian violet.

Antibiotic susceptibility tests

Disk resistance tests. All strains were sensitive to the metronidazole (5 µg), neomycin (1000 µg), kanamycin (1000 µg) and penicillin (2 units) disks, except *B. gracilis* ATCC 33236 which was resistant to penicillin. Two ulcer strains, seven urethritis strains, five dental strains, *B. gracilis* ATCC 33236 and *W. recta* NCTC 11489 were resistant to the rifampicin disks.

MIC determinations with all strains except *W. recta* NCTC 11489 are summarised in table III. All strains were resistant to vancomycin (MIC > 16 mg/L) and teicoplanin (64–> 128 mg/L). All the ulcer and urethritis isolates and the reference strains of *B. ureolyticus* and *B. gracilis* were resistant to nalidixic acid (8–128 mg/L) but eight of the dental strains were more sensitive (2–4 mg/L). All the clinical isolates and *B. ureolyticus* NCTC 10941 were sensitive to benzylpenicillin (MIC ≤ 0.03–0.12 mg/L) but the MIC for the two *B. gracilis* strains was 2.0 mg/L. Polymyxin MIC values were 1.2 or 2.4 i.u./ml for the reference strains and most clinical isolates, but two ulcer strains and three

dental strains required 4.8 i.u./ml for inhibition and one ulcer strain was inhibited by 0.6 i.u./ml.

GLC analysis

Determination of the VFA end-products of metabolism proved to be difficult and unrewarding. Many strains did not grow well in broth media, hence the use of agar cultures. Most strains were tested after incubation in all three broth media and on the three agar media. VFA were not detected in any cultures in *B. ureolyticus* broth and in only four cultures on *B. ureolyticus* agar and two on FAA. Small amounts, sometimes only trace amounts, of acetic, propionic, *iso*-valeric and, from two strains, *n*-butyric acids were detected in 15 cultures in FAB, six out of 24 cultures tested in FAB + supplements, and 11 cultures on blood agar. The results obtained with cultures of the same strain on different media were not necessarily the same; VFA were detected in FAB cultures but not blood-agar cultures of 10 strains and in blood-agar cultures but not FAB cultures of seven strains. These equivocal results were spread throughout the test groups and VFA production data were, therefore, discarded from further analyses.

Pyrolysis-mass spectrometry

A Py-MS ordination diagram representing the separation of the test strains in the first three discriminant functions (representing 72% of inter-strain variance) shows clear separation of strains into several clusters, most of which comprise strains from a single source group (fig. 1). A dendrogram representing Py-MS inter-strain discrimination based upon all 30 discriminant functions (representing 100% of inter-strain variance) is shown in fig. 2. Eight groups (A–H) were evident. One dental strain was an extreme outlier (group J) and was not included in further analyses. The separation into eight groups (plus the outlier) was one of the optimal

Table III. Antibiotic susceptibility of *B. ureolyticus* isolates and comparative strains

Strains	n	Minimum inhibitory concentration (mg/L)											
		vancomycin			teicoplanin			benzylpenicillin			nalidixic acid		
		MIC50	MIC90	Range	MIC50	MIC90	Range	MIC50	MIC90	Range	MIC50	MIC90	Range
Ulcer	15	>16	>16	>16	>128	>128	128– >128*	≤0.03	≤0.03	≤0.03	16	32	8–128*
Urethritis	12	>16	>16	>16	>128	>128	>128†	≤0.03	<0.12	≤0.03	16	32	16– >128†
Dental	14	>16	>16	>16	>128	>128	64– >128†	0.12	0.12	0.06– 0.12†	4.0	64	2.0– 128†
<i>B. ureolyticus</i> NCTC 10941	1			>16			>128			<0.03			16
<i>B. gracilis</i>	2			>16			>128			2.0			16
													1.2– 2.4

*Only 14 ulcer strains were tested.
†Only 11 urethritis strains were tested.
‡Only 13 dental strains were tested.

Fig. 1. Ordination diagram representing the position of the test strains defined by the first three canonical discriminant functions. The numbers on the axes represent distances in inter-strain discrimination based upon χ^2 df 29 probabilities that the strains were identical. $\square$ = ulcer strains; $\circ$ = urethritis strains; $\diamond$ = dental strains; $\star$ = reference strains.

solutions indicated in Normix cluster analysis. Within the largest group, B (26 strains), up to seven sub-groups could be distinguished (D^2 c. 12). Three groups contained only single strains—an ulcer strain (group H), a dental strain (group D) and *B. gracilis* ATCC 33236 (group A)—and group F contained only two strains, *B. gracilis* WAL 85/253C and a dental strain.

Two Py-MS groups contained strains from only one clinical group—five ulcer strains in group E and four dental strains in group G. The other Py-MS groups contained strains from different clinical sources. Within group B, subgroups B5 and B6 each contained a single strain, subgroups B1 and B7 each contained two ulcer strains, and subgroup B4 an ulcer and a urethritis strain. Subgroup B3 contained four dental strains and the largest subgroup, B2 (14 strains), contained *B. ureolyticus*

NCTC 10941, two ulcer strains, eight urethritis strains and three dental strains.

Comparison of Py-MS with conventional grouping

Simple matching UPGMA analysis of the data from biochemical, antibiotic susceptibility and tolerance tests produced the dendrogram shown in fig. 3. The cophenetic correlation of this approach was 0.89906. Five main groups (I–V) were delineated. Except for one dental strain which clustered as an outlier of group II (subgroup IIe), there was clear separation between dental strains and those from other sources. The other 13 dental strains formed two clusters, groups I (6 strains) and III (5 strains), with two quite dissimilar strains making up group V. The two *B. gracilis* strains formed group IV. All the ulcer and urethritis strains formed

Fig. 2. Dendrogram representing inter-strain discrimination in Py-MS. C strains = ulcer isolates; U strains = urethritis isolates; D strains = dental isolates.

a closely linked group, II; they could be separated into four subgroups (IIa–d) by differences in one or two test results; strains in each subgroup had identical test results. The largest subgroup, IIa, contained 17 strains—eight each from ulcers and urethritis plus *B. ureolyticus* NCTC 10941. Subgroup IIb contained three ulcer and two urethritis strains and subgroup IIc contained four ulcer strains and one from urethritis. The separation

of the dental strains into three groups was based on overall differences in results with the set of tests used and did not correlate specifically with the results of the oxidase and urease tests, generally regarded as important tests for *B. ureolyticus* and related species. A comparison of the groups delineated by UPGMA analysis of the data from conventional tests and the Py-MS groups is shown in table IV.

Fig. 3. Dendrogram derived from simple matching UPGMA analysis of the data from biochemical, antibiotic susceptibility and tolerance tests. C strains = ulcer isolates; U strains = urethritis isolates; D strains = dental isolates.

Discussion

From the results of these varied approaches to characterisation it can be seen that the anaerobic gram-negative corroding bacilli form a heterogeneous group but that there is a strong correlation between site of isolation and strain characters; isolates associated with superficial ulcers and urethritis formed a homogeneous set of strains that represents a reliable species description consistent with the reference pattern of *B. ureolyticus*. These

findings are in agreement with the reports of Fontaine *et al.* (1986) and Taylor *et al.* (1986, 1987) that the urethritis strains were *B. ureolyticus* and were indistinguishable from *B. ureolyticus* strains from other sources. Dental isolates were mostly different from *B. ureolyticus* and were heterogeneous amongst themselves. *B. gracilis* reference strains were included because of similarities with *B. ureolyticus* but they remained distinct from all the groups of clinical isolates in Py-MS and conventional tests.

Table IV. Comparison of groups obtained by conventional tests and Py-MS

Py-MS group	Biochemical group				
	II	III	I	IV	V
H	1				
C	3				
E	5				
B	20	3	3		
G		2	2		
F			1	1	
A				1	
D					1
J					1

II=ulcer and urethritis isolates (C strains and U strains) and NCTC 10941; I, III, V=dental isolates (D strains); IV=*B. gracilis*.

Pitting growth on primary isolation is the character by which all strains included in this study were selected and it remains the initial character for the recognition of this group of organisms in the clinical laboratory, but as others have reported (Sutter *et al.*, 1985) it is an unreliable character during laboratory manipulation and may be lost by adaptation to artificial media during subculture. Similarly, a simple test of motility (microscopy of wet films), which was used because of its applicability in a clinical laboratory, did not provide a reliable differential criterion. *W. recta*, an oral organism associated with some forms of periodontal disease (Tanner *et al.*, 1981), was included in some tests for comparison with dental isolates. It was the only reference strain that was actively motile. Although this type of test may be considered unreliable for anaerobes (Willis and Phillips, 1988), the consistent motility of *W. recta* provided a reference standard for comparison with test strains, but many results were equivocal. Attempts to demonstrate motility in semi-solid agar were not successful and it is clear that electronmicroscopy studies are needed to determine whether the strains that gave equivocal results possess flagella.

The two distinct approaches to characterisation used in this study, conventional tests and Py-MS, produced similar groupings. Both distinguished between a single main cluster of strains from ulcers

and urethritis (and the *B. ureolyticus* reference strain) and several distinct clusters from dental sources and *B. gracilis*. The ulcer-urethritis strains and *B. ureolyticus* NCTC 10941 gave consistent results, but the dental strains gave varied patterns, and even within the small number of variable conventional tests (oxidase, urease, nitrate, motility, nalidixic acid resistance, ethyl violet tolerance) they gave various combinations of results with no consistent association between the tests. The dental strains were divided into two single-member groups (D, V and J, V) and two larger groups (B3, G; I, II) by Py-MS and conventional methods but the two approaches differed in the allocation of strains to the two larger groups. This reflects the difficulty of comparing the results of such different classification approaches. The dendrograms obtained by analysis of the Py-MS and conventional data differ in their ordering of the clusters, which is a result of problems of representing differences in these two sets of characteristics in a hierarchical diagram. Dendrograms and ordination diagrams reveal only a proportion of the structure of the similarity matrix—the cophenetic correlations were 0.9 for the dendrogram based on conventional tests and 0.87 for the Py-MS dendrogram, and the ordination diagram represented only 72% of inter-strain variance. The representation of Py-MS data in the dendrogram (fig. 2) gave incomplete separation of the ulcer-urethritis strains and the dental strain. In particular one distinct sub-cluster of four dental strains, D31–34, came within Py-MS group B (B3) and a cluster of five ulcer strains was separated as group E. However, the similarity matrix for Py-MS data, from which the dendrogram was derived, showed group E to be similar to group B, which supports the general conclusion of this study that *B. ureolyticus* isolates from ulcers and urethritis form a single species group, different from the dental isolates which form several distinct groups that might represent different species, as yet undefined.

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REFERENCES

- Aries R E, Gutteridge C S, Ottley T W 1986 Evaluation of a low-cost, automated pyrolysis-mass spectrometer. *Journal of Analytical and Applied Pyrolysis* **9**: 81–98.
- Crawford R M M, Wishart D 1968 A rapid classification and ordination method and its application to vegetation mapping. *Journal of Ecology* **56**: 385–404.
- Duerden B I 1983 The Bacteroidaceae: *Bacteroides*, *Fusobacterium*, and *Leptotrichia*. In: Wilson G S *et al.* (eds) *Topley and Wilson's Principles of bacteriology, virology and immunity*, 7th edn, vol. 2. Edward Arnold, London, pp 114–136.
- Duerden B I, Bennett K W, Faulkner J 1982 Isolation of *Bacteroides ureolyticus* (*B. corrodens*) from clinical infections. *Journal of Clinical Pathology* **35**: 309–312.

- Duerden B I, Collee J G, Brown R, Deacon A G, Holbrook W P 1980 A scheme for the identification of clinical isolates of gram-negative anaerobic bacilli by conventional bacteriological tests. *Journal of Medical Microbiology* **13**: 231–245.
- Duerden B I, Goodwin L, O'Neil T C A 1987 Identification of *Bacteroides* species from adult periodontal disease. *Journal of Medical Microbiology* **24**: 133–137.
- Duerden B I, Holbrook W P, Collee J G, Watt B 1976 The characterization of clinically important gram-negative anaerobic bacilli by conventional bacteriological tests. *Journal of Applied Bacteriology* **40**: 163–188.
- Eiken M 1958 Studies on an anaerobic rod-shaped gram-negative microorganism: *Bacteroides corrodens* n.sp. *Acta Pathologica et Microbiologica Scandinavica* **43**: 404–416.
- Fontaine E A R, Borriello S P, Taylor-Robinson D, Davies H A 1984 Characteristics of a gram-negative anaerobe isolated from men with non-gonococcal urethritis. *Journal of Medical Microbiology* **17**: 129–140.
- Fontaine E A R, Bryant T N, Taylor-Robinson D, Borriello S P, Davies H A 1986 A numerical taxonomic study of anaerobic gram-negative bacilli classified as *Bacteroides ureolyticus* isolated from patients with non-gonococcal urethritis. *Journal of General Microbiology* **132**: 3137–3146.
- Fontaine E A R, Taylor-Robinson D, Hanna N F, Coufalik E D 1982 Anaerobes in men with urethritis. *British Journal of Venereal Diseases* **58**: 321–326.
- Hawkins D A, Fontaine E A R, Thomas B J, Boustouller Y L, Taylor-Robinson D 1988 The enigma of non-gonococcal urethritis: role for *Bacteroides ureolyticus*. *Genitourinary Medicine* **64**: 10–13.
- Henriksen S D 1948 Studies in gram-negative anaerobes: gram-negative anaerobic rods with spreading colonies. *Acta Pathologica et Microbiologica Scandinavica* **25**: 368–375.
- Holbrook W P, Ogston S A, Ross P W 1978 A method for the isolation of *Bacteroides melaninogenicus* from the human mouth. *Journal of Medical Microbiology* **11**: 203–207.
- Huff S M, Meuzelaar H L C, Pope P L, Kjeldsberg C R 1981 Characterisation of leukemic and normal white blood cells by Curie-point pyrolysis-mass spectrometry. I. Numerical evaluation of the results of a pilot study. *Journal of Analytical and Applied Pyrolysis* **3**: 95–109.
- Jackson F L, Goodman Y E 1972 Transfer of the facultatively anaerobic organism *Bacteroides corrodens* Eiken to a new genus, *Eikenella*. *International Journal of Systematic Bacteriology* **22**: 73–77.
- Jackson F L, Goodman Y E 1978 *Bacteroides ureolyticus*, a new species to accommodate strains previously identified as "*Bacteroides corrodens anaerobic*". *International Journal of Systematic Bacteriology* **28**: 197–200.
- Jones B M, Geary I, Alawattagama A B, Kinghorn G R, Duerden B I 1985 *In-vitro* and *in-vivo* activity of metronidazole against *Gardnerella vaginalis*, *Bacteroides* spp. and *Mobiluncus* spp. in bacterial vaginosis. *Journal of Antimicrobial Chemotherapy* **16**: 189–197.
- Khairat O 1967 *Bacteroides corrodens* isolated from bacteraemias. *Journal of Pathology and Bacteriology* **94**: 29–40.
- Labbé M, Hansen W, Schoutens E, Yourassowsky E 1977 Isolation of *Bacteroides corrodens* from human clinical specimens. Comparative study of incidence and methods of identification. *Infection* **5**: 159–162.
- Magee J T, Hindmarch J M, Bennett K W, Duerden B I, Aries R E 1989 A pyrolysis mass spectrometry study of fusobacteria. *Journal of Medical Microbiology* **28**: 227–236.
- Masfari A N, Kinghorn G R, Duerden B I 1983 Anaerobes in genitourinary infections in men. *British Journal of Venereal Diseases* **59**: 255–259.
- Masfari A N, Kinghorn G R, Hafiz S, Barton I G, Duerden B I 1985 Anaerobic bacteria and *Herpes simplex* virus in genital ulceration. *Genitourinary Medicine* **61**: 109–113.
- Paster B J, Dewhirst F E 1988 Phylogeny of campylobacters, woliniellas, *Bacteroides gracilis*, and *Bacteroides ureolyticus* by 16S ribosomal ribonucleic acid sequencing. *International Journal of Systematic Bacteriology* **38**: 56–62.
- Reinhold L 1966 Untersuchungen an *Bacteroides corrodens* (Eiken 1958). *Zentralblatt für Bakteriologie Originale A* **201**: 49–57 (in German).
- Rotimi V O, Faulkner J, Duerden B I 1980 Rapid methods for the identification of clinical isolates of gram-negative anaerobic bacilli. *Medical Laboratory Sciences* **37**: 331–339.
- SPSSX User's Guide 1983 McGraw-Hill Book Co., New York.
- Schroter G, Stawru J 1970 Die Bedeutung von *Bacteroides corrodens* Eiken 1958 in Rahman der Tonsillenflora. *Zeitschrift für Medizinische Mikrobiologie und Immunologie* **155**: 241–247 (in German).
- Shah H N, Collins M D 1983 Genus *Bacteroides*: a chemotaxonomical perspective. *Journal of Applied Bacteriology* **55**: 403–416.
- Shah H N, Collins M D 1988 Proposal for reclassification of *Bacteroides asaccharolyticus*, *Bacteroides gingivalis*, and *Bacteroides endodontalis* in a new genus, *Porphyromonas*. *International Journal of Systematic Bacteriology* **38**: 128–131.
- Sneath P H A 1957 Some thoughts on bacterial classification. *Journal of General Microbiology* **17**: 184–200.
- Sokal R R, Michener C D 1958 A statistical method for evaluating systematic relationships. *University of Kansas Science Bulletin* **38**: 1409–1438.
- Sutter V L, Citron D M, Edelstein M A C, Finegold S M 1985 Wadsworth anaerobic bacteriology manual, 4th edn. Star Publishing Co., Belmont, CA.
- Tanner A C R, Badger S, Lai C-H, Listgarten M A, Visconti R A, Socransky S S 1981 *Wolinella* gen. nov., *Wolinella succinogenes* (*Vibrio succinogenes* Wolin et al.) comb. nov., and description of *Bacteroides gracilis* sp. nov., *Wolinella recta* sp. nov., *Campylobacter concisus* sp. nov., and *Eikenella corrodens* from humans with periodontal disease. *International Journal of Systematic Bacteriology* **31**: 432–445.
- Taylor A J, Costas, M, Owen R J 1987 Numerical analysis of electrophoretic protein patterns of *Bacteroides ureolyticus* clinical isolates. *Journal of Clinical Microbiology* **25**: 660–666.
- Taylor A J, Dawson C A, Owen R J 1986 The identification of *Bacteroides ureolyticus* from patients with non-gonococcal urethritis by conventional biochemical tests and by DNA and protein analysis. *Journal of Medical Microbiology* **21**: 109–116.
- Ward J H 1963 Hierarchical grouping to optimize an objective function. *Journal of the American Statistical Association* **58**: 236–244.
- Wideman P A, Citronbaum D M, Sutter V L 1977 Simple disk technique for detection of nitrate reduction by anaerobic bacteria. *Journal of Clinical Microbiology* **5**: 315–319.
- Williams R A D, Bowden G H, Hardie J M, Shah H N 1975 Biochemical properties of *Bacteroides melaninogenicus* subspecies. *International Journal of Systematic Bacteriology* **25**: 298–300.
- Willis A T, Phillips K D 1988 Anaerobic infections; clinical and laboratory practice. Public Health Laboratory Service, London.
- Wishart D 1982 Clustan 2 user manual. Edinburgh University Press, Edinburgh.
- Wolfe J H 1970 Pattern clustering by multivariate mixture analysis. *Multivariate Behavioral Research* **5**: 329–350.