The differentiation of asaccharolytic anaerobic gram-positive cocci by protein electrophoresis

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Summary. Conventional biochemical and antibiotic sensitivity tests were used to allocate 87 clinical isolates of anaerobic gram-positive cocci to currently recognised species, in comparison with type and other authentic reference strains. Whole-cell protein electrophoresis was then performed with extracts of each strain. Allowing for difficulties of standardisation, it was possible to allocate most of the organisms to species-related groups on the basis of protein patterns. Organisms identified conventionally as *Peptostreptococcus anaerobius* and *P. micros* formed homogeneous groups by protein electrophoresis. There was evidence for heterogeneity amongst strains identified as *P. asaccharolyticus* (two groups, including *P. indolicus*), *P. prevotii* and *P. magnus*. However, aberrant *P. prevotii* strains were allocated to the *P. asaccharolyticus* groups, leaving a homogeneous *P. prevotii* group, and if *P. variabilis* were re-instated as a species, the remaining *P. magnus* strains could be divided into two groups. Of the anaerobic gram-positive cocci in the National Collection of Type Cultures deposited by Hare, Group IV is *P. magnus*, Group IX is *P. micros* and Groups I, III and VIII appear to be related to the butyrate-producing species *P. asaccharolyticus* and *P. prevotii*, but are strongly saccharolytic.

Introduction

The taxonomy of the anaerobic gram-positive cocci is still in a state of evolution. There have been major changes in the last two editions of Bergey's Manual, from the scheme described by Rogosa¹ to that now proposed by Holdeman Moore *et al.*² for peptococci and peptostreptococci, and by Hardie³ for anaerobic streptococci. The changes are largely based on more extensive biochemical studies and determination of G+C contents of DNA, and to a certain extent on DNA-DNA homology studies^{4,5} supported by analysis of the structure of mucopeptide.⁶ Although some species are now well established, others are conceded to be heterogeneous.²

Schemes for the identification of these organisms have been limited by the changes in taxonomy and by the fact that anaerobic cocci from clinical material are usually unreactive, especially in tests for carbohydrate fermentation. Recently-introduced commercial kits have included a wider variety of biochemical tests, and species differentiation has improved, but there are still problems, as, for example, with the differentia-

tion of the indole-negative, butyrate-producing cocci,⁷ complete failure to identify an important minority of strains,⁸ and a lack of an independent and authoritative identification of the clinical strains used in such systems.

Numerical taxonomy based on patterns of whole-cell proteins separated by polyacrylamide gel electrophoresis is a recognised tool in microbial systematics.^{9,10} Cato *et al.*¹¹ demonstrated distinct differences in the patterns obtained with anaerobic cocci and were able to distinguish *Peptostreptococcus micros* from the then *Peptococcus magnus*, a distinction previously based largely on cell size. They suggested that the technique might be valuable for identification as well as in taxonomy.

The present study was designed to assess the usefulness of whole-cell protein electrophoretic patterns in the identification of asaccharolytic gram-positive cocci commonly associated with human disease.

Materials and methods

Reference strains

The reference strains that were used are listed in table I. Recognised saccharolytic species were included to study the relatedness of the asaccharolytic and saccharolytic species and of the anaerobic gram-positive cocci lodged in the National Collection of

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Type Cultures (NCTC) as the Hare Groups, many of which were reported to be saccharolytic by Hare.¹² Members of Hare Group VIa and VIIb proved to be micro-aerophilic on re-examination; therefore, they were not included in this study. Similarly, members of Hare Group V were excluded, as the representative strains were found to be gram-negative.

Clinical strains

A collection of anaerobic gram-positive cocci, isolated from various clinical specimens examined in the Department of Microbiology, St Thomas' Hospital, London was also included. Strains were selected to include organisms originating from skin (27 strains), faeces (16), urogenital (27) and oral (17) specimens. Great care was taken to select single colonies for subculture to ensure purity.

Storage and maintenance

During the investigation, strains were maintained on non-selective blood-agar plates, subcultured at 48-h intervals and incubated anaerobically at 37°C. A modification of the method of Feltham *et al.*¹³ was used for long-term storage of all organisms at -70°C in a medium containing sterile defibrinated horse blood 10% and sterile glycerin 5% in thioglycollate broth (Oxoid CM23).

Incubation

All cultures were incubated in a mixture of N₂ 80%, CO₂ 10%, H₂ 10% in an anaerobic chamber at 37°C.

Conventional identification of clinical isolates

Antibiotic susceptibility tests, selected from those recommended by Sutter and Finegold,¹⁴ Watt *et al.*¹⁵ and Wren *et al.*¹⁶ were used for presumptive identification.

Two horse blood-agar plates (Oxoid CM 331) were inoculated with sufficient of the test organism to produce semi-confluent growth. Disks containing penicillin G 2 u, kanamycin 1000 µg, vancomycin 5 µg and colistin 10 µg, supplied on a ring (Mast Laboratories, M/ID8), were applied to one plate and single disks of metronidazole 2.5 µg and novobiocin 5 µg were applied to the other. Zones of inhibition were measured after incubation anaerobically for 24 or 48 h. The zones around the Mast ring were interpreted according to the manufacturer's instructions, based on the recommendations of Sutter and Finegold.¹⁴ Zones > 26 mm in diameter were interpreted as indicating sensitivity to metronidazole and of > 10 mm diameter as indicating sensitivity to novobiocin.^{16, 17}

Biochemical tests

These were based on methods recommended by Holdeman, Cato and Moore.¹⁸

Carbohydrate fermentation. Tests for fermentation (acid production) were performed in 3-ml volumes of Fastidious Anaerobe Broth (FAB, Lab M: code 71) containing 1% of glucose, fructose or mannose (added as a filter-sterilised 10% solution to the cooled, autoclaved medium). A heavy suspension of a 24- or 48-h culture of the test organism harvested from a blood-agar purity plate was made in 3 ml of FAB without added carbohydrate, and three drops of this suspension were used to inoculate each test carbohydrate. The cultures were incubated anaerobically for at least 48 h or until no obvious increase in growth (assessed as turbidity) had occurred for 24 h. The pH of each culture was measured with a Corning-Eel pH meter Model No. 7 and the change in pH was compared with that of an inoculated, incubated culture in FAB without added carbohydrate. All the strains tested produced a heavy turbid suspension in this broth after incubation for a maximum of 3 days.

The API 20A identification kit (Analytab Products Inc.) was used to examine any isolate that fermented any of the three carbohydrates tested by the conventional method.

Indole production and nitrate reduction. The tests were performed by the methods of Cowan¹⁹ in 3-ml volumes of Indole-Nitrate Medium (BBL 11299) inoculated with the test organism and incubated anaerobically at 37°C for at least 48 h.

Gas chromatography

Analysis of volatile fatty acid end-products of glucose metabolism was performed on 2-4-day cultures of each organism in FAB medium with glucose 1% w/v by the method of Gargan and Phillips.²⁰ A Pye Unicam GCD chromatograph equipped with a flame ionisation detector was used to analyse the samples. A 10-µl volume of the culture supernate was injected into a 1.5 m × 0.4 mm glass column (Pye Unicam 9435-179-18051), packed with Chromosorb 101 (Applied Science Labs. Inc. 04825). Results were recorded on a Philips 8251 flat-bed recorder. The following settings were used for the detection of volatile and non-volatile fatty acids: column temperature 200°C, injector and detector temperature 240°C and O₂-free N₂ carrier-gas flow rate 40 ml/min. With this method the lower limits of detection were 0.1 mmol/ml except for lactic acid (0.4 mmol/ml) and succinic acid (4 mmol/ml).

Whole-cell protein electrophoresis

A loopful of growth from a 24- or 48-h culture on blood agar was suspended in two 50-ml volumes of freshly prepared pre-reduced broth containing yeast extract 10%, sodium thioglycollate 0.5%, sodium

chloride 2.5%, sodium bicarbonate 0.4%, glucose 1.0% and Tween 80 0.01% in Proteose Peptone No. 3 (Difco 0122-01-2). The cultures were incubated anaerobically for 24 or 48 h to obtain a heavy suspension of organisms. Purity was checked by subculture of one loopful of each 48-h broth culture on a non-selective horse-blood-agar medium, which was then incubated anaerobically for 5 days and examined carefully for the presence of contaminants. After centrifugation at approximately 3000 rpm, the deposit was washed twice in distilled water, and resuspended in 1 ml of lysozyme (Sigma L-6876) 0.1% in pH 6.0 buffer and incubated at 37°C for 3 h. After lysis, 0.1 ml of sodium dodecyl sulphate (SDS) 20% was added to each preparation and the mixture was placed in a boiling water bath for 2 min. The suspension was then centrifuged at 12 000 rpm for 30 min in an Anderman 5414 centrifuge and 1.0 ml of the supernate was added to 0.2 ml of sample buffer containing 0.1625 M Tris-HCl, pH 6.8, SDS 2%, glycerol 10% and 2-mercaptoethanol 5%.

Proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) in 10% gels as described by Jackman.¹⁰ The separation gel was prepared by adding a mixture of 67 ml of monomer (acrylamide 30%, bis acrylamide 2.7%), 50 ml of separation buffer (1.5 M Tris-HCl, pH 8.8) and distilled water (to 193 ml) to 2 ml of SDS 10%, ammonium persulphate 90 mg in 5 ml of distilled water and TEMED 60 µl. The total volume was thus 200 ml. The stacking gel contained the same ingredients in different amounts: 10 ml of monomer, 15 ml of buffer and distilled water to 54 ml; 0.6 ml of SDS 10%, ammonium persulphate 60 mg in 5 ml of distilled water and TEMED 40 µl. The tank buffer consisted of 0.25 M Tris, pH 8.3, 0.192 M glycine and SDS 0.1%.

Samples of 100 µl were placed in the wells, along with bromophenol blue in some samples, and a constant current of 30 mA was applied in a Bio Rad 165-1572 Multi-6 gel protean cell with a Pharmacia EPS 500/400 solid power supply, until the tracker dye had travelled 100 mm from the stacking gel-separation gel interface. The gels were then removed and stained in Coomassie Blue 0.125% overnight and destained in acetic acid 10% plus methanol 25% in distilled water until the background was clear. Gels were then dried in a slab gel drier (LKB 2003).

A Vitatron TLD 100 scanning densitometer (MSE Instruments) was used to scan each gel. The following settings were used: tungsten lamp, red filter 603, slit width 0.25 mm, slit length 3 mm, scanning speed 1 mm/s. The densitometer was attached to a Commodore PET 3032 microcomputer and 3040 disk unit via an Ortholog 181 analogue-digital converter (Ortholog Ltd). The computer programs used for analysis were as described by Jackman *et al.*²¹ The first program accepted from the converter 10 voltage values per mm of gel scanned. The second program searched the first 50 points of each scan, and accepted the highest value as the beginning of the gel, and ignoring the next mm

so as to exclude the gel start peak, averaged the subsequent 1000 points in groups of 10, so that each electrophoretic pattern was represented by 100 values. The third program compared the traces, searched for best fit, and formed a similarity matrix based on the correlation coefficient of Sneath and Sokal.²² The fourth program applied the unweighted pair group method using averages (UPGMA) of Sneath and Sokal²² to cluster the similarity matrix generated by the third program, and the dendrogram was then plotted.

The gels were also examined visually for evidence of major differences or similarities within and between conventionally identified species and computer-derived groups. The findings were confirmed by independent observation by the three authors.

Results

Conventional tests

All named reference strains produced expected results in conventional tests (table I). However, it was noted that the type strain of *P. micros* was resistant to novobiocin, although Wren *et al.*¹⁶ had previously reported that this species was sensitive on the basis of tests on two clinical isolates. This observation was taken into account in identification of clinical isolates and the Hare Group organisms.

The reactions of the Hare Group organisms in conventional tests are also shown in table I. Only two of the 10 organisms examined could be identified to species level, Hare group IV (NCTC nos. 9804 and 9815) as *P. magnus*. Strains NCTC 9811 and 9821 were asaccharolytic and could not be allocated to species. The six remaining strains were saccharolytic and produced large amounts of butyric acid from glucose metabolism. Although some strains fermented several carbohydrates they could not be identified by the schemes outlined by Holdeman Moore *et al.*² or by Hardie.³

The provisional identification of the 87 clinical isolates and their reactions in conventional biochemical tests are shown in table II. All these organisms were asaccharolytic. A characteristic volatile fatty acid profile was used to identify 19 strains as *P. anaerobius*. Fifteen indole-positive butyrate-producing strains were identified as *P. asaccharolyticus* and the indole-negative butyrate-positive strains were identified as *P. prevotii*. Thirty-four butyrate-non-producing strains were speciated purely by cell size and colonial appearance. Thirty strains were identified as *P. magnus* because their cell size was >0.6 mm and four were identified as *P. micros* because they were small and had a colonial appearance similar to that of the type strain of *P. micros*. The other three butyrate-non-producing novobiocin-resistant isolates were not allocated to species.

Table I. Reference strains of anaerobic cocci and their important biochemical reactions

Reference strains and numbers as received		Results in tests for				
		coagulase	glucose fermentation	indole production	nitrate reduction	volatile fatty acid production
Hare Group organisms*						
NCTC 9801	Hare Group I	—	+	+	—	A, p, B
NCTC 9803	Hare Group III	—	+	+	—	A, p, B
NCTC 9814	Hare Group III	—	+	+	—	A, p, B
NCTC 9804	Hare Group IV	—	—	—	—	A
NCTC 9815	Hare Group IV	—	—	—	—	A
NCTC 9808	Hare Group VIIa	—	+	—	—	A, p, B
NCTC 9810	Hare Group VIII	—	+	—	—	A, p, B
NCTC 9820	Hare Group VIII	—	+	—	—	A, p, B
NCTC 9811	Hare Group IX	—	—	—	—	A
NCTC 9821	Hare Group IX	—	—	—	—	A
Named and reference strains†						
ATCC 27337	<i>P. anaerobius</i>	—	w	—	—	A, p, ib, b, IC, l, s
NCIB 10074	<i>P. asaccharolyticus</i> (ATCC 14963)‡	—	—	+	—	A, p, B, l, s
ATCC 29427	<i>P. indolicus</i>	+	—	+	+	A, p, B
ATCC 11088	<i>P. indolicus</i>	+	—	+	+	A, p, B
ATCC 15794	<i>P. magnus</i>	—	—	—	—	A, l, s
V5464-1	<i>P. micros</i> (ATCC 33270)§	—	—	—	—	A, l, s
NCIB 10768	<i>P. prevotii</i> (ATCC 14952)‡	—	—	—	—	A, p, B, l, s
ATCC 14953	<i>P. saccharolyticus</i>	—	+	—	+	A, l
ATCC 27731	<i>Peptococcus niger</i>	—	—	—	—	A, p, ib, B, iv, C, l, s
V0546	<i>Str. parvulus</i> (ATCC 33793)§	—	+	—	—	A, l, s
ATCC 27340	<i>Str. productus</i>	—	+	—	—	A, p, l, s

Numbers in parenthesis are ATCC numbers of strains received from other sources.

A, acetic; B, butyric; IB, isobutyric; C, caproic; IC, isocaproic; L, lactic; S, succinic; IV, isovaleric.

Upper case, large amounts; lower case, small amounts.

* Obtained from the National Collection of Type Cultures, Central Public Health Laboratory, 61 Colindale Avenue, London.

† Obtained from the American Type Culture Collection, Rockville, Maryland 20852, USA, except where otherwise stated.

‡ Obtained from the National Collection of Industrial Bacteria, Torry Research Station, Aberdeen.

§ Obtained from Professor L. V. Holdeman, Blacksburg, VA, USA.

Table II. Biochemical reactions of clinical isolates of anaerobic cocci

Provisional identification	Number of isolates	Biochemical reactions				Volatile fatty acids produced
		Co	GI	In	Ni	
<i>P. anaerobius</i>	19	—	—	—	—	A, p, ib, iv, IC, l, s
<i>P. asaccharolyticus</i>	15	—	—	+	—	A, p, B, l, s
<i>P. magnus</i>	30	—	—	—	—	A, p, l, s
<i>P. micros</i>	4	—	—	—	—	A, p, l, s
<i>P. prevotii</i>	16	—	—	—	—	A, p, B, l, s
Unidentified novobiocin resistant strains*	3	—	—	—	—	A, p, l, s

Co, coagulase; GI, glucose fermentation; In, indole production; Ni, nitrate reduction.

Volatile fatty acids: see table I.

* Not clearly morphologically *P. micros*.

Protein electrophoresis: computerised analysis

Fig. 1 shows a dendrogram based on the numerical analysis of protein patterns of 108 strains of anaerobic cocci, including 87 clinical isolates and 21 reference strains (including four type strains) by the correlation coefficient and UPGMA clustering.

No single similarity level divided the strains into completely satisfactory groups but four broad groups (A–D) were defined at a similarity of 0.30, and several

distinct clusters were found within each group. These will be described in detail.

Group A. This group contained 41 organisms and was dominated by strains identified conventionally as *P. anaerobius* and *P. asaccharolyticus*. Eighteen of the 19 strains of *P. anaerobius* examined, including the type strain ATCC 27337, and 12 of the 15 strains of *P. asaccharolyticus* examined, including the type strain ATCC 14963 (NCIB 10074), fell into this group. Four strains conventionally identified as *P. prevotii*, two

Fig. 1. Dendrogram based on electrophoretic protein patterns. The similarity coefficient was the correlation coefficient (r) and UPGMA clustering was used. The best fit between patterns was found by calculation of r at lateral shifts of up to 2 mm between patterns.

strains identified as *P. magnus*, and three other reference strains—*P. indolicus* ATCC nos. 11088 and 29427 and Hare Group III NCTC 9814—were also found in Group A.

Group B. This group contained 36 strains and was very complex, but discrete clusters were found that merit particular attention.

Cluster B1 contained three strains identified as *P. prevotii*, two strains identified as *P. asaccharolyticus*, Hare Group III strain NCTC 9803 and Hare Group I strain NCTC 9801. These organisms reacted similarly in conventional tests. All produced butyrate as a major

fatty-acid end-product of glucose metabolism. *P. prevotii* and *P. asaccharolyticus* were distinguished on the basis of indole production only, and both *P. asaccharolyticus* and the two Hare Group organisms fermented various carbohydrates and gave different reactions in the tests for hydrolysis of substrates by pre-formed enzymes.

Cluster B2 contained the three clinical isolates and the type strain of *P. micros* (V5464). The electrophoretic protein patterns of these strains had an average correlation of 0.90.

Two clinical isolates in cluster B3 were identical (765·3 and 61·4), but differed from all other strains. The rest of the organisms in this cluster were mostly known to belong to quite different species.

Cluster B4 contained nine clinical strains identified as *P. prevotii*, reference strains *P. prevotii* ATCC 14952 (NCIB 10768) and Hare Group VIII (NCTC 9810), and one clinical isolate identified only as a *Peptostreptococcus* species. Strain NCTC 9810 was biochemically similar to *P. prevotii* as it was indole negative and produced butyrate from glucose metabolism, but it fermented several carbohydrates, whereas *P. prevotii* is asaccharolytic.

Group C. This group contained 23 organisms including three reference strains identified as *P. magnus*—the type strain of the species, ATCC 15794, and two Hare Group IV organisms (NCTC nos 9804 and 9815)—and 18 of the 30 clinical isolates identified as *P. magnus*. One clinical isolate identified as *P. asaccharolyticus* (59·7) and the type strain of *Streptococcus parvulus* were also placed in Group C.

Group D. This group contained only clinical isolates identified as *P. magnus*.

Protein electrophoresis: visual assessment

The results of the assessment of a combination of

the results of conventional tests, computer-derived Groups, and visual examination of gels (fig. 2) are shown in table III.

The 19 clinical strains identified by conventional means as *P. anaerobius*, including one clinical isolate (622·2) that was placed by computer analysis in Group B despite the clear similarity of its gel to those of strains in Group A, and the type strain ATCC 27337, all had a similar protein pattern characterised by a single dominant high mol.-wt band. They formed a single combined Group Aa.

Of the 15 isolates identified conventionally as *P. asaccharolyticus*, 12 had patterns similar to that of the type strain NCIB 10074 (ATCC 14963), characterised by a pair of high mol.-wt bands. These, with four strains conventionally identified as *P. prevotii* on the basis of a negative indole reaction, two reference strains of *P. indolicus* and a saccharolytic Hare Group III strain (NCTC 9814) clustered into computed Group A and all were separated visually from *P. anaerobius* strains into combined Group Ab. The remainder of computed Group A was made up of two clinical isolates conventionally identified as *P. magnus* that resembled each other but were distinct from any of the type or reference strains.

Two clinical strains identified as *P. asaccharolyticus*, two reference strains of saccharolytic Hare Groups I

Fig. 2. Protein electrophoretic patterns of: 1, *P. anaerobius* ATCC 27337 (type strain); 2, *P. asaccharolyticus*—possibly homology group 1, NCIB 10074, ATCC 14953 (type strain); 3, *P. indolicus* ATCC 11088; 4, possible new taxon 1 (clinical isolate); 5, *P. asaccharolyticus*—possibly homology group 2; 6, *P. micros* V5464-1, ATCC 33270 (type strain); 7, possible new taxon 2 (clinical isolate); 8, possible *P. prevotii* NCIB 10768, ATCC 14952 (reference strain); 9, *P. magnus* ATCC 15794 (type strain), 10, *P. magnus*-like (clinical isolate); 11, possible *P. variabilis* (clinical isolate).

Table III. Allocation of strains to groups on the basis of conventional biochemical tests, and numerical analysis combined with visual inspection of protein electrophoretic patterns

Species	Type strain	Reference strains	Conventional identification of clinical isolates (number)	Protein electrophoresis	
				computed groups	combined groups
<i>P. anaerobius</i>	ATCC 27337		<i>P. anaerobius</i> (18) <i>P. anaerobius</i> (1)	A B	Aa Aa
<i>P. asaccharolyticus</i> homology group 1	NCIB 10074	Hare III NCTC 9814 <i>P. indolicus</i> ATCC 11088 ATCC 29427	<i>P. asaccharolyticus</i> (12) <i>P. prevotii</i> (4)	A A	Ab Ab
Possible new taxon 1	...	...	<i>P. magnus</i> (2)	A	Ac
<i>P. asaccharolyticus</i> possibly homology group 2	...	Hare I NCTC 9801 ^s	<i>P. asaccharolyticus</i> (2) <i>P. prevotii</i> (3)	B1 B1	Ba Ba
		Hare III NCTC 9803 ^s		B1	Ba
<i>P. micros</i>	V5464-1	(Hare IX NCTC 9821) (Hare IX NCTC 9811)	<i>P. micros</i> (3)	B2	Bb
Possible new taxon 2	...	...	<i>Peptostreptococcus</i> spp (2)	B3	Bc
Possible <i>P. prevotii</i>	...	NCIB 10768 Hare VIII NCTC 9810 ^s Hare VIII NCTC 9820 ^s Hare VIIa NCTC 9808 ^s	<i>P. prevotii</i> (9)	B B B B	Bd Bd Bd Bd
<i>P. magnus</i>	ATCC 15794	Hare IV NCTC 9804	(0)	C	Ca
<i>P. magnus</i> -like	...	Hare IV NCTC 9815	<i>P. magnus</i> (18)	C	Cb
Possible <i>P. variabilis</i>	...	...	<i>P. magnus</i> (8)	D	Da
Unallocated*			(8)		

s, Saccharolytic strains.

* Unallocated strains were: clinical isolates *P. magnus* (2; 730-5, 547-5), *Peptostreptococcus* sp. (1; 634-9), *P. asaccharolyticus* (1; 59-7) (conventional identification); *Pc. niger*, ATCC 27731; *Str. productus*, ATCC 27340; *Str. parvulus*, V0546; *P. saccharolyticus*, ATCC 14953.

(NCTC 9801) and III (NCTC 9803) and three strains originally identified as *P. prevotii* made up combined Group Ba.

Three clinical isolates of *P. micros* and the type strain V5464 (ATCC 33270) came in computed Group B as a single cluster B2. A Hare Group IX organism (NCTC 9821), falling into computed Group B4, appeared similar visually, and the five organisms were therefore allocated to combined Group Bb. On the basis of visual inspection, the Hare Group IX organism NCTC 9811 could also belong to combined Group Bb.

Two unidentified clinical isolates (new taxon 2) formed a separate group both by computation and visually (combined Group Bc).

Of the 16 isolates provisionally identified as *P. prevotii*, nine of the 12 not clustering with *P. asaccharolyticus* had patterns resembling a reference strain (ATCC 14952, NCIB 10768). Unfortunately the type strain of *P. prevotii* was lost during the study. One Hare Group VIII strain (NCTC 9810) fell into the same cluster (Bd) and two more were added on the basis of visual inspection (Hare Group VIII, NCTC 9820; Hare Group VIIa NCTC 9808). The Hare Group IX strain NCTC 9821, although in this computed Group, was visually in Group Bb (see above).

In computed Group C, only the type strain of *P. magnus* and a Hare Group IV strain (NCTC 9804) had the same protein pattern on visual inspection (Ca). Eighteen other *P. magnus* strains and a Hare Group IV strain (NCTC 9815) clustered into a separate but contiguous group, and visually were very similar to each other (Cb).

Group D was made up entirely of clinical isolates identified as *P. magnus* (Da), and all were similar visually.

Eight strains, including four reference strains not expected to find a place, were not allocated by the combination of computerised and visual analysis. Three of the reference strains (*Pc. niger* ATCC 27731, *P. productus* ATCC 27340 and *P. saccharolyticus* ATCC 14953) and three clinical isolates (730-5, 547-5 and 634-9) fell into computed Group B3, and the other two organisms (*Str. parvulus* V546 and clinical isolate 59-7) were allocated to computed Group C.

Discussion

The groups created by the numerical analysis alone were governed, to a certain extent, by various confounding factors, some of which proved impossible

to overcome. Some groups of strains possessed large amounts of one or two proteins with the same mol. wt, and this character was over-weighted by the correlation coefficient used. This was the major problem with the species *P. anaerobius* and *P. asaccharolyticus*, and resulted in the grouping together of the members of these two species. Furthermore, the limitations of the similarity coefficient used and difficulties with the reproducibility of the electrophoresis affected the numerical analysis. We therefore concluded that a numerical analysis qualified by visual assessment, provided the most acceptable taxonomic groups.

This combination of visual comparison and numerical analysis of whole-cell protein patterns resulted in a more satisfactory taxonomic structure which, when comparison was possible, largely endorsed the findings of the few other large chemotaxonomic studies on the Peptococcaceae,⁴⁻⁶ and of the only other studies of proteins of selected anaerobic cocci that we have been able to find.^{11,23}

P. anaerobius

We found, in agreement with Ezaki *et al.*,⁴ Huss *et al.*⁵ and Smith *et al.*,²³ that the species *P. anaerobius* is homogeneous and distinct from the other asaccharolytic, anaerobic gram-positive cocci. This species can be easily and accurately identified by its characteristic volatile fatty-acid pattern as well as by protein electrophoresis. Like Smith *et al.*,²³ who examined only surface proteins, we found that most isolates had a single major intense protein band. Most of our isolates came from the vagina or miscellaneous skin and soft tissue infections.

The butyrate-producing cocci

We concluded that differentiation of *P. prevotii* and *P. asaccharolyticus* on the basis only of indole production is unreliable. The species *P. asaccharolyticus*, as currently defined, is heterogeneous. We distinguished two groups which appear to correspond to two of the three homology groups distinguished by Ezaki *et al.*,⁴ each containing clinical isolates identified conventionally as *P. asaccharolyticus* or *P. prevotii*.

We agree with Huss *et al.*⁵ and with Murdoch *et al.*⁷ that *P. indolicus* is closely related to *P. asaccharolyticus*—indeed we were unable to differentiate the protein patterns of two reference strains of *P. indolicus* from those of the type strain of *P. asaccharolyticus* ATCC 14953. The strains of Hare Groups I and III were allocated to the second homology group—both Weiss⁶ and Huss *et al.*⁵ relate them to *P. asaccharolyticus*. Most of our isolates in putative homology Group 1 came from the female genital tract, while those from putative homology Group 2 came from more varied sites.

The species *P. prevotii* appears to be homogeneous on the basis of protein electrophoresis, but we were not able to include the type strain (ATCC 9321) in our

final analysis although we did include a reference strain (ATCC 14952). However, this strain may not be typical of *P. prevotii* as it is now described by Holdeman Moore *et al.*² On the basis of visual inspection we also allocated three saccharolytic Hare Group organisms (VIIa, NCTC 9808; VIII, NCTC nos. 9810 and 9820) to this group. Both Weiss⁶ and Huss *et al.*⁵ related Hare Group VIII to *P. prevotii*. Our isolates of "*P. prevotii*" came from various sites, including the vagina. Smith *et al.*²³ found that their *P. prevotii* was heterogeneous but, as we did originally, could have included indole-negative *P. asaccharolyticus* strains.

P. micros

We concluded that the species *P. micros* is homogeneous and, in agreement with Huss *et al.*,⁵ that Hare Group IX organisms probably belong to this species. *P. micros* has been previously reported to be sensitive to novobiocin,¹⁶ but in our experience, the type strain, all clinical isolates and both Hare Group IX organisms were resistant. It has now been suggested that *P. micros* can be differentiated from *P. magnus* by its strong alkaline phosphatase activity¹⁸ and by the production of a wider range of aminopeptidases.⁷ Our three clinical strains came from pelvic pus, a liver abscess and a paronychia.

P. magnus

We concluded, as did Smith *et al.*,²³ that, on the basis of protein patterns, *P. magnus*, as currently defined, is heterogeneous. In agreement with Weiss⁶ and Huss *et al.*,⁵ we found that the Hare Group IV organisms are *P. magnus*. Cato *et al.*¹¹ reported that the protein patterns of the type strains of *P. magnus* and *P. variabilis* were quite distinct, although the species *P. variabilis* was no longer recognised and had become a synonym of *P. magnus*. In our study, the protein patterns of the strains identified as *P. magnus* fell into two distinct groups and the second of the groups, Da, was characterised by a protein pattern that greatly resembled that published by Cato for the species *P. variabilis*. Unfortunately, the type strain of the previously recognised species *P. variabilis* was not included in our study, and we were unable to find any conventional test that could be used to distinguish between the two "species". Most of the isolates in the *P. magnus*-related groups came from skin infections (axilla, paronychia, breast, sebaceous cyst etc.) and only one came from the vagina.

The Hare Groups

The Hare Groups of anaerobic cocci¹² deposited with the NCTC continue to be a puzzle even when the facultatively anaerobic organisms have been excluded. Protein electrophoresis contributed to the elucidation of some of the problems. We agree with others that

Groups I and III are related to *P. asaccharolyticus*⁶ but cannot agree with Huss *et al.*⁵ that they resemble *P. prevotii* once indole-negative *P. asaccharolyticus* strains have been removed from that species, or with Hare¹² himself that Group I is *S. putridus* and that Group III is *Staphylococcus aureus*.

We believe that Hare Group III (NCTC 9814) is closely related to the *P. asaccharolyticus* homology group 1 of Ezaki *et al.*,⁴ but since it is saccharolytic we cannot allocate it definitely to that group. The second Hare Group III organism (NCTC 9803) and Hare Group I (NCTC 9801), both saccharolytic strains, are closely related accordingly to Weiss⁶ and belong to a second group of *P. asaccharolyticus* which we believe may be homology group 2 of Ezaki *et al.*⁴

One Hare Group IV strain (NCTC 9804) was clearly *P. magnus*, but the other belonged to a *P. magnus*-like group which otherwise contained only clinical isolates. This result is compatible with those of Weiss⁶ and Huss *et al.*⁵ and of Hare¹² himself who believed that the two organisms were of different species.

Although the protein patterns of Hare Groups VIIa and VIII appeared similar to the eye, only Group VIII, NCTC 9810 could be related convincingly to the group that we called *P. prevotii*, in agreement with Huss *et al.*⁵ Again, the strains were saccharolytic and the true grouping could be more in relation to *P. tetradius* (*Gaffkya anaerobia*) of Ezaki *et al.*⁴ but we made no direct comparisons.

Both Hare Group IX organisms, which are asaccharolytic, were related to *P. micros* although the protein patterns were not identical. In this we agree with Weiss⁶ and Huss *et al.*,⁵ but not with Hare¹² who

thought that they could be *Staph. anaerobius*, a synonym of *P. magnus*.

In addition to the recognised species we found evidence for two new taxonomic groups, each made up of two clinical isolates. The organisms in one group were identified conventionally as *P. magnus*, but differed greatly in protein patterns. The two clinical isolates in the other new group were identified conventionally only as *Peptostreptococcus* sp. and were allocated by the computer to Group B3.

One area of the dendrogram, the greater part of computed Group B3 was completely unsatisfactory. It contained the unrelated organisms *Pc. niger*, *P.* (now *Str.*) *productus*, *P.* (now *Staph.*) *saccharolyticus*, one Hare Group IX strain (NCTC 9811) and three clinical isolates, none of which had protein patterns like any of the reference strains. These organisms require further study.

Whole-cell protein electrophoresis is a relatively easy-to-perform investigation that examines a large proportion of the products of the bacterial genome. However, practical problems affect the reproducibility of the technique and make numerical analysis of the protein patterns difficult. Furthermore, anaerobic gram-positive cocci are difficult organisms to work with, and problems encountered in this study with the standardisation and reproducibility of sample preparation exacerbated the difficulties of numerical analysis. Despite these problems, visual comparison of the protein patterns showed that the differences between many of the strains tested was large, and if the practical problems can be overcome, numerical analysis of whole-cell protein patterns has great potential.

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