Classification of oral pigmented anaerobic bacilli by pyrolysis mass spectrometry and biochemical tests

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Summary. Clinical (66) and reference (5) strains of pigmented gram-negative anaerobic bacilli, identified as *Prevotella intermedia* (47), *Pr. melaninogenica* (1), *Pr. corpora* (8), *Porphyromonas asaccharolyticus* (12), *P. endodontalis* (1) and *P. gingivalis* (2), were examined by pyrolysis mass spectrometry (PMS) and in conventional tests. Numerical classification based on conventional test reaction patterns (CTRPs) resolved five clusters, four comprising strains identified as *Pr. intermedia*, *Pr. corpora*, *Pr. melaninogenica*, and *P. gingivalis* respectively, and one comprising strains identified as *P. asaccharolyticus* and *P. endodontalis*. Numerical classification based on PMS showed a similar division, with decreasing homogeneity of chemical composition in the order *Pr. intermedia*, *Pr. corpora*, *P. asaccharolyticus*, which agreed with the order of homogeneity in CTRPs. PMS clusters corresponding to the genus *Porphyromonas* were clearly distinct from those of the genus *Prevotella*. PMS and CTRP classification disagreed on cluster membership for six strains. PMS identification from blind challenge sets was in agreement with conventional identification for 64 of 67 strains.

Introduction

Gram-negative anaerobic bacilli that produced black or brown colonies on blood-containing media were first described by Oliver and Wherry¹ and called *Bacterium* (later *Bacteroides*) *melaninogenicus*. This species encompassed all pigmented bacteroides but was subsequently divided into three subspecies.² Subsequently the asaccharolytic strains were removed, first to a distinct species, *B. asaccharolyticus*,³ later as a subgroup of species within the *Bacteroides*⁴ and then to a separate genus, *Porphyromonas*, comprising three species, *P. asaccharolyticus*, *P. endodontalis* and *P. gingivalis*.⁵ The saccharolytic pigmented strains remained members of the melaninogenicus-oralis group of *Bacteroides* which included pigmented and non-pigmented strains; but these too were later separated from the genus *Bacteroides sensu stricto*⁶ (formerly the fragilis group of *Bacteroides*) as a new genus, *Prevotella*.⁷ The pigmented *Prevotella* spp. comprise *Pr. intermedia*, *Pr. melaninogenica*, *Pr. corpora*, *Pr. loescheii* and *Pr. denticola*. These changes were largely the result of DNA homology studies.

Pigmented bacteroides are members of the normal flora of the mouth,^{8–10} vagina¹¹ and colon,¹² and are also implicated in a range of purulent and necrotising

infections at these sites.^{13–17} In the mouth they are found in the gingival crevice in both health and disease.⁹ *Pr. melaninogenica* is part of the normal commensal gingival flora⁹ and appears to be of little pathological significance,¹⁰ whereas *Pr. intermedia* is more commonly associated with acute necrotising gingivitis and chronic periodontal disease.^{18–20} *P. gingivalis* is linked with severe, rapidly progressive periodontitis and generalised juvenile periodontal disease.^{15,21} *P. asaccharolyticus* has not been associated with oral colonisation or disease, but in a study of the gingival flora in patients with adult periodontal disease²² we isolated large numbers of pigmented strains, about half of which appeared asaccharolytic in rapid tests,^{23–25} produced small amounts of n-butyric acid, and were identified as *P. asaccharolyticus*. Most of the saccharolytic strains were identified as *Pr. intermedia*. Similar results were obtained in a subsequent longitudinal study of adult periodontal disease.²⁶ The work described here was done partly because of doubts as to the validity of these identifications as *P. asaccharolyticus*, based solely on rapid tests.

Pyrolysis mass spectrometry (PMS) is a rapid characterisation method that has been used in classification, identification and typing of micro-organisms.^{27,28} The spectra produced reflect cell chemical composition as 'fingerprint' or pattern data with a complex statistical structure. Analysis of these data requires multivariate statistical strategies. However, modern statistical program suites available for micro- and mini-computers can be used to reduce raw

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spectral data to statistically meaningful strain similarities, for classification, for typing, and to identify spectra of unknown strains by comparison with a concurrently produced database of spectra for named strains. The approach is particularly relevant in studies of genera where characterisation is difficult because strains are "biochemically unreactive" or require prolonged incubation to yield readable results.²⁸ The identification problem described above seemed a fitting application, which would also extend our investigations into traditional and PMS numerical classification of the anaerobic gram-negative bacilli.²⁹⁻³¹

Materials and methods

Bacterial strains

The 71 strains of pigmented gram-negative anaerobic bacilli tested comprised dental (53), vaginal (4) and wound (9) isolates, together with the type strains *Pr. intermedia* NCTC 9336, *P. asaccharolyticus* NCTC 9337, *P. gingivalis* NCTC 11834, *P. endodontalis* HG 370 and the collection strain *P. gingivalis* W50. These were preserved at -70°C in glycerol 15% broth, and cultured on BM agar³² at 37°C in an atmosphere of H_2 10%, N_2 80%, CO_2 10% for 48 h before testing.

Conventional tests

The strains were examined in a range of conventional tests, only 10 of which gave reproducible results relevant in differentiation. These were: production of acid from glucose, lactose and sucrose;^{24,25} production of indole;^{24,25} α -fucosidase, α -glucosidase and β -N-acetyl glucosaminidase;³³ agglutination of sheep erythrocytes;³⁴ and production of n-butyric and phenyl acetic acids,³⁵ detected by gas-liquid chromatography.³⁶ Tests which proved non-discriminatory or irreproducible were: tolerance to sodium taurocholate, gentian violet and Victoria blue 4R; resistance to penicillin (2 units), neomycin (1000 μg), kanamycin (1000 μg) and rifampicin (15 μg) disks;²³⁻²⁵ gas-liquid chromatography for non-volatile fatty acids;³⁶ and fluorescence under UV illumination.^{4,37}

Conventional identification was based on comparison of conventional test reaction patterns (CTRP) with those of type strains of the currently accepted species. The UPGMA hierarchical clustering strategy³⁸ and simple matching coefficient (S_{sm})³⁹ were used to produce a numerical classification based on the 10 tests listed above, which gave the dendrogram shown in fig. 1.

Pyrolysis mass spectrometry

PMS methods and analysis strategies were as described previously.^{29-31,40} Briefly, the 71 strains, plus two duplicates (one each of *Pr. intermedia* and *P. asaccharolyticus*) were assigned blind numeric codes. Four replicate foils were prepared for each of these 73

"strains". The replicate foils of each strain were divided between four batches, each with a different randomised strain sequence. The four batches of foils were analysed over 2 days on a PYMS 200X automated pyrolysis mass spectrometer (Horizon Instruments, Heathfield, Sussex). Immediately before each batch run, six foils of three strains selected at random were pyrolysed and the spectra were discarded. In our experience, this procedure limits excess irreproducibility in spectra produced early in a batch run.

The blind coding of the 73 "strains" examined was not broken until PMS classification was completed. In this study, discriminant analysis based on intensities of masses 50–120 yielded 11 canonical discriminant function axes (CDFAs) describing 84.6% of inter-strain discrimination. Spectral co-ordinates on these CDFAs were processed in UPGMA hierarchical clustering to produce a dendrogram (fig. 2). The squared Euclidian distance similarity coefficient (D_E) used is an estimate of the χ^2 probability (10 dof) that spectra of the strains compared were statistically indistinguishable.²⁸

PMS blind identification was performed as previously^{28-31,40} by inter-species discriminant analysis of normalised intensities for masses 50–120. Unduly optimistic estimates of identification error rates are likely to be obtained if spectra of the strain to be identified are included in the characterisation database.²⁸ To avoid this, strains were sorted by conventional species identification, being put into the appropriate species set in the order of their initial random numerical blind coding. The strains within each species set were then numbered sequentially and two identification analyses were performed. In the first, all spectra of strains assigned odd species set numbers constituted the teaching set, describing the species characteristics in PMS, and all spectra of the even numbered strains were the blind challenge set. In the second analysis, membership of these sets was reversed. Spectra for the single strains representing *Pr. melaninogenica*, *P. endodontalis* and *P. gingivalis* were omitted from these analyses, because no valid challenge sets could be assembled. Spectra of the six strains showing disagreement between PMS and conventional classification were included only in the challenge sets in both analyses. PMS species identifications for each spectrum in the challenge sets were noted, along with the consensus of the results for the four replicate spectra of each strain. If the identification results for a spectrum indicated a possible second choice at $p > 5\%$, the result was noted as equivocal and was ignored in the consensus strain identification.

Results

Conventional classification

At an S_{sm} of 75–80%, widely regarded as indicative of species discrimination, five clusters were resolved

Fig. 1. A dendrogram representing a numerical classification of the strains based on conventional test reaction patterns. Tight correlation between conventional identification and cluster membership was expected, as the same tests contributed to both.

(fig. 1). Clusters I–III were distinct from clusters IV and V at $S_{sm} = 0.5$. All strains identified as *Prevotella* spp. were found in clusters I–III and all strains identified as *Porphyromonas* spp. were found in clusters IV and V. Cluster I comprised all 47 strains identified conventionally as *Pr. intermedia*, including the type strain NCTC 9336. Cluster II comprised a single strain, identified as *Pr. melaninogenica*. Cluster III comprised all eight strains identified as *Pr. corpora*.

Cluster IV comprised all 12 strains identified as *P. asaccharolyticus*, including the type strain NCTC 9337. The strain of *P. endodontalis* (ATCC 35406) was a single member outlier of this group. Cluster V comprised both strains identified as *P. gingivalis* (type strain NCTC 11834 and collection strain W50). Cluster I showed tight homogeneity of CTRPs, clusters III and IV were more diverse. Tight correlation between cluster membership and conventional identification was expected, because the same test results contributed to both.

PMS classification

Pairs for the two duplicated cultures were close neighbours ($D_E < 80$) in the dendrogram (fig. 2). Thirteen clusters were resolved at $D_E = 600$. Cluster A comprised 46 strains, 44 of which were identified conventionally as *Pr. intermedia*, including the type strain NCTC 9336, and two as *Pr. corpora*. The single member cluster B comprised the strain identified as *Pr. melaninogenica*. Cluster C comprised two strains, one

identified as *Pr. intermedia* and the other as *Pr. corpora*. Clusters D and E comprised five strains identified as *Pr. corpora*. Clusters F and G each comprised a single strain identified as *Pr. intermedia*. These clusters contained all strains identified as *Prevotella* spp. and were clearly distinct from the remaining clusters containing *Porphyromonas* spp.

Clusters H–J comprised the 12 strains identified as *P. asaccharolyticus* including the type strain NCTC 9337. Cluster K comprised the single strain of *P. endodontalis* (ATCC 35406), and clusters L and M comprised the collection strains of *P. gingivalis* W50 and NCTC 11834. Only six strains showed anomalous PMS clustering in comparison with conventional results; these are discussed below.

Comparison of PMS and conventional classification results

Of the six strains clustered by PMS in disagreement with conventional identification and classification results, three, identified conventionally as *Pr. corpora* in cluster III, were found in PMS sub-groups A, A and C respectively. The remaining three, identified as *Pr. intermedia* in cluster I, clustered in PMS groups C, F and G respectively.

The level of CTRP diversity within each species and within the CTRP classification clusters was mirrored in the PMS classification. With the exceptions listed above, PMS group A showed tight homogeneity of chemical composition, corresponding to the tight

Fig. 2. A dendrogram representing a numerical classification of the strains based on pyrolysis mass spectrometry data. Cluster composition (conventional identification, number of strains) was: A, *Pr. intermedia* 44 plus *Pr. corpora*, 2; B, *Pr. melaninogenica*, 1; C, *Pr. intermedia*, 1 plus *Pr. corpora*, 1; D and E, *Pr. corpora*, 4 and 1 respectively; F and G, *Pr. intermedia*, 1 and 1 respectively; H–J, *P. asaccharolyticus*, 8, 3 and 1 respectively; K, *P. endodontalis*, 1; L and M, *P. gingivalis*, 1 and 1 respectively.

homogeneity of CTRPs for the *Pr. intermedia* strains of the equivalent conventional cluster I. Conventional cluster III (*Pr. corpora*) corresponded to the more diverse PMS clusters D and E, and showed wider diversity of CTRPs. *P. asaccharolyticus* showed a still wider CTRP diversity, corresponding to the wide divergence of PMS clusters H–J.

PMS identification

Of the 67 strains identified in PMS, 95.5% yielded consensus results in agreement with conventional identification. Only three (4.5%) showed disagreement. All three showed anomalous clustering in the PMS classification; two strains identified conventionally as *Pr. corpora* but clustering in PMS group A were identified as *Pr. intermedia*, and one strain identified conventionally as *Pr. intermedia* but clustering in PMS group C was identified as *Pr. corpora*. The two strains comprising PMS clusters F and G were identified as *Pr. intermedia*, in agreement with conventional identification, despite their anomalous clustering in PMS. Of the 280 individual spectra examined, 262 (93.6%) were identified unequivocally in agreement with conventional identification, 14 (5%) were identified to a species in disagreement with conventional identification (these included the four replicate spectra for each of the three strains identified in disagreement), three (1.1%) gave equivocal identifications, with either the first or second choice

species in agreement with conventional identification, and one (0.3%) gave an equivocal identification with neither first nor second choice species in agreement.

Discussion

Clusters in the CTRP and PMS classifications were clearly identified with the currently recognised species and both classifications indicated major differences between the genera *Prevotella* and *Porphyromonas*. At species level, strains identified as *Pr. corpora* and as *P. asaccharolyticus* showed higher levels of compositional and biochemical heterogeneity than was found for the majority of strains identified as *Pr. intermedia*. However, the success in PMS identification indicated a cohesive core of compositional properties characteristic of each of these species.

By contrast, the two strains of *P. gingivalis* differed widely in PMS, but yielded identical results in conventional tests. Examination of a larger collection of *Porphyromonas* strains might resolve this problem, and throw light on the position of *P. endodontalis*, which appeared similar to *P. asaccharolyticus* in CTRPs and PMS.

There was over 90% agreement between PMS identification and that based on CTRPs, as in previous studies of *Fusobacterium*,²⁹ coryneforms⁴⁰ and the “*Streptococcus milleri* group”.⁴¹ The only disagreement was in identification as *Pr. corpora* or *Pr.*

intermedia. Conventional discrimination of these species can be difficult, and either CTRP or PMS identifications may have been erroneous for the few "mis-identified" strains. All strains identified as *P. asaccharolyticus* by rapid methods in our previous studies of oral flora^{22,26} were identified as *P. asaccharolyticus* by PMS, and none showed anomalous clustering in the PMS and CTRP classifications. The PMS identification results confirm that each of these three species shows a distinctive core of characteristic compositional features.

The possibility of exploiting PMS as a rapid identification system for gram-negative anaerobes seems attractive. The speed, throughput and disposables costs of PMS are suited to such an application. Wieten and his colleagues have outlined the successful use of PMS in routine identification for clinical isolates of mycobacteria.^{42,43} Because the long-term reproducibility of PMS remains poorly documented, they included a teaching set of named strains of mycobacteria with each batch of unidentified isolates, an approach which they term "operational fingerprinting".⁴²

The biochemically unreactive nature of these organisms limited the number of tests used in the CTRP classification to 10. A far greater number of tests is recommended for numerical classification, and at least 30 tests showing discriminatory reactions are normally found for the more tractable aerobic genera of bacteria. With a larger number of tests, inter-strain similarity estimates are less affected by test errors, and tend to a stable figure, affected little by inclusion or exclusion of individual test results. The failure to find adequate numbers of discriminatory conventional tests has limited the application of numerical classification to the bacteroides and the progress in dividing this group into phenotypically and genotypically homogeneous species.

Clearly, reliable prediction of pathogenesis and other ecological properties is unlikely to be attained in a classification based on heterogeneous species. The increasing complexity of the classification of the

bacteroides may be irksome, but it is essential for rational work on pathogenesis. If the salmonellas and klebsiellas were viewed as one species and the shigellas and pseudomonads as another, there would be little hope of making sense of their pathogenicity. The bacteroides are probably as diverse as the enterobacteria, requiring a classification of similar complexity.

The introduction of techniques for genotypic classification of the bacteroides has allowed progress, but the low throughput of these techniques severely limits the number of strain comparisons that can be made. Often, only one or two strains are taken to represent a single species in such investigations. Phenotypic resemblance to these marker strains in a limited number of conventional tests may not be a reliable guide to genotypic homogeneity, raising the possibility that the currently postulated species structure may be incomplete. Although the results here do not suggest such anomalies, in a concurrent study of the non-pigmented bacteroides³¹ several species appeared heterogeneous and two undescribed centres of variation were found.

The present throughput limitations of genetic methods suit them more to confirmation of phenotypic classifications than to exploratory work. What is required is a high throughput technique which can be applied to anaerobes and examines a large number of characters. Objective numerical studies for large strain collections would then give a rational framework for DNA hybridisation work. PMS is rapid, automated, with a high throughput and low running costs, and examines the large number of characters that contribute to total cell composition. Our work with other genera^{30,31,40} has shown good correlations between PMS and CTRP classifications, and with DNA-DNA hybridisation data.⁴¹ PMS seems an approach well suited to the current needs of those interested in the taxonomy of anaerobes.

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