Multivariate analysis of quantitative chemical and enzymic characterization data in classification of *Actinobacillus*, *Haemophilus* and *Pasteurella* spp.

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Chemotaxonomic data for strains of *Actinobacillus*, *Haemophilus* and *Pasteurella* spp. were analysed using three multivariate statistical strategies; principal components, partial least squares discriminant, and soft independent modelling of class analogy. The species comprised *Actinobacillus actinomycetemcomitans*, *Haemophilus aphrophilus*, *H. paraphrophilus*, *H. influenzae*, *Pasteurella multocida*, *P. haemolytica* and *P. ureae*. Strains were characterized by cell sugar and fatty acid composition, lysis kinetics during EDTA and EDTA plus lysozyme treatment, and methylene blue reduction. In total 23 quantitative variables were compiled from chemotaxonomic analyses of 25 strains. *A. actinomycetemcomitans* and *H. aphrophilus* formed distinct classes which differed from those of *H. paraphrophilus*, *H. influenzae* and *Pasteurella* spp. All characterization variables, except those describing fatty acid content, contributed significantly to inter-species discrimination.

Introduction

The genera *Actinobacillus*, *Haemophilus* and *Pasteurella*, which constitute the family Pasteurellaceae, are attracting renewed interest amongst clinical microbiologists and taxonomists (Brondz & Olsen, 1986a). This group of organisms is isolated more frequently than previously and from clinical sources hitherto considered unusual. The *Actinobacillus*–*Haemophilus*–*Pasteurella* group represents a spectrum of taxonomically similar organisms which are difficult to differentiate by classical procedures. The distinction between *A. actinomycetemcomitans* and *H. aphrophilus* has been questioned and the inclusion of *A. actinomycetemcomitans* in the genus *Haemophilus* has been proposed (Kilian, 1976; Potts *et al.*, 1985). In addition, the distinction between *H. aphrophilus* and *H. paraphrophilus* has been put into doubt (Tanner *et al.*, 1982). It has been suggested that the current classification of recognized actinobacilli and

pasteurellas does not enable the two genera to be distinguished, and DNA homology studies show that several species assigned to the genus *Pasteurella* are more closely related to the genus *Actinobacillus* (Mutters *et al.*, 1984).

Chemotaxonomy is the application of chemical methods to the classification and identification of micro-organisms; it is based on the analysis of particular substances in whole cells or parts of cells, or of metabolic products (Brondz & Olsen, 1986b). Cell constituents and enzymes have been used previously in taxonomic studies of the *Actinobacillus*–*Haemophilus*–*Pasteurella* group (Brondz & Olsen 1984, 1985a, b, c, 1989; Olsen & Brondz, 1985). In this study, we subjected data sets obtained previously to principal component (PC) analysis (Jolliffe, 1986; Wold *et al.*, 1987), partial least squares (PLS) discriminant analysis (Geladi & Kowalski, 1986), and soft independent modelling of class analogy (SIMCA) (Wold, 1976) to determine whether multivariate pattern recognition methods can be used in the chemotaxonomy of organisms of the *Actinobacillus*–*Haemophilus*–*Pasteurella* group. Such methods have been used previously to classify *Neisseria* (Sørensen *et al.*, 1985), *Porphyromonas* (Brondz *et al.*, 1989a) and fungi (Blomquist *et al.*, 1979; Brondz *et al.*, 1989b).

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Abbreviations: PC, principal component; PLS, partial least squares; SIMCA, soft independent modelling of class analogy; HEWL, hen egg white lysozyme.

Methods

Bacteria. The 25 bacterial strains tested (Table 1) comprised the species *A. actinomycetemcomitans*, *H. aphrophilus*, *H. paraphrophilus*, *H. influenzae* type b, *P. haemolytica*, *P. multocida* and *P. ureae*. Strains were obtained directly from the American Type Culture Collection (ATCC; Rockville, USA), the National Collection of Type Cultures (NCTC; London, UK), the Forsyth Dental Center (FDC; Boston, USA) and the Royal Dental College, Aarhus, Denmark. The identification of the clinical strains was based on physiological characteristics and end-products (acid and gas) of metabolism (Tanner *et al.*, 1982; Kilian, 1976).

Growth and harvesting. Stock cultures were stored in liquid nitrogen. Growing cultures were maintained anaerobically (80% N₂/10% H₂/10% CO₂) on blood or chocolate agar plates, and transferred weekly. For chemotaxonomic analyses, organisms were cultivated in Brain Heart Infusion (Difco) broth in air plus 10% CO₂ for 5 d at 37 °C. To grow the *H. paraphrophilus* and *H. influenzae* strains, the broth was supplemented with filter-sterilized NAD (1 mg l⁻¹) and haemin (5 mg l⁻¹). For chromatographic analyses the organisms were harvested by centrifugation, washed three times in deionized distilled water and

lyophilized over phosphorous pentoxide. For lysozyme assays, blood or chocolate agar plates inoculated with *Actinobacillus*, *Haemophilus* and *Pasteurella* spp. were incubated anaerobically for 2 d. Colonies were transferred to preheated Brain Heart Infusion broth and cultured anaerobically for 15½ h. For *H. paraphrophilus* and *H. influenzae* type b strains, the broth was supplemented with NAD and haemin.

Bacteriolysis. After preparation of hen egg white lysozyme (HEWL) (Jolles *et al.*, 1962), bacteriolysis in Tris/maleate buffer (0.005 M, pH 7.2) supplemented with EDTA (0.01 M) or EDTA (0.01 M)/HEWL (1.0 µg ml⁻¹) was recorded using a Hitachi model 100-200 spectrophotometer. The OD₅₄₀ was measured after incubation at 37 °C for 5, 10, 20, 30, 40 or 50 min. Before measuring, the optical density of the bacterial suspensions had been adjusted to 0.6 (approximately 9.1 × 10⁸ cells ml⁻¹) and 20 µl of buffer or lysozyme solution had been added to the spectrophotometric cuvettes, each containing the bacteria suspended in 1980 µl 0.005 M-Tris/maleate buffer supplemented with 0.01 M-EDTA.

Methylene blue reduction. This was assessed by observing colour changes developing in bacterial cell suspensions to which the redox indicator methylene blue had been added (Brondz & Olsen, 1985b).

Table 1. *Bacteria investigated*

Strain no.	Species and strain designation*	Source†	Site of origin
<i>A. actinomycetemcomitans</i>			
1	33384 (9710)*	ATCC (NCTC)	Lung abscess
2	2112	FDC	Periodontitis
3	511	FDC	Periodontitis
4	N27	FDC	Periodontitis
5	29524	ATCC	Chest aspirate
6	29523	ATCC	Blood
7	29522	ATCC	Mandibular abscess
8	2097	FDC	Periodontitis
9	2043	FDC	Periodontitis
10	Y4	FDC	Periodontitis
<i>H. aphrophilus</i>			
11	33389 (5906)*	ATCC (NCTC)	Endocarditis
12	19415	ATCC	Endocarditis
13	13252	ATCC	
14	655	FDC	Periodontitis
15	654	FDC	Periodontitis
16	626	FDC	Periodontitis
17	621	FDC	Periodontitis
<i>H. paraphrophilus</i>			
18	29241*	ATCC	Paronychia
19	29240	ATCC	Parietal abscess
25	29242	ATCC	Trachea
<i>H. influenzae</i>			
20	33533	ATCC	Blood
21	31441	ATCC	Clinical isolate
<i>P. multocida</i>			
22	10322*	NCTC	
<i>P. haemolytica</i>			
23	9380*	NCTC	
<i>P. ureae</i>			
24	10219*	NCTC	Ozena

* Type strain.

† ATCC, American Type Culture Collection; NCTC, National Collection of Type Cultures; FDC, Forsyth Dental Center.

Recordings were made every 5 min during incubation at 37 °C, under air protection, using a standard colour scale.

Methanolysis and derivatization. Whole lyophilized cells were methanolysed with 2 M-HCl in anhydrous methanol for 24 h at 85 °C. The methanolysates were dried with a stream of nitrogen gas and derivatized in a 1:1 (v/v) mixture of acetonitrile/trifluoroacetic acid anhydride at 90 °C for 3 min (Brondz & Olsen, 1984, 1985a).

Gas chromatography. A type 5880 gas chromatograph (Hewlett-Packard) fitted with an electronic integrator was used. Samples were analysed on a Chrompack CP-Sil 5 (polydimethylsiloxane) glass capillary column (25 m × 0.22 mm i.d.; film thickness 0.14 µm). Identities of the methanolysed and derivatized sugars and fatty acids were determined by co-chromatography with authentic standards and by gas chromatography-mass spectrometry. Individual components were quantified from their peak areas and adjusted according to their relative molar responses.

Statistical analyses. This study reports on the distribution of cell fatty acids (four variables) and sugars (four variables) together with reduction of the redox indicator methylene blue (one variable) and bacteriolysis by EDTA and EDTA/HEWL (14 variables). Accordingly, a total of 23 quantitative variables have been compiled from chemotaxonomic analyses of 25 strains. The variables were as follows: lysis in EDTA within 5 min (variable 1); within 10 min (2); within 15 min (3); within 20 min (4); within 30 min (5); within 40 min (6); within 50 min (7); lysis in EDTA/HEWL within 5 min (variable 8); within 10 min (9); within 15 min (10); within 20 min (11); within 30 min (12); within 40 min (13); within 50 min (14); methylene blue reduction (min) (variable 15); galactose (%) (variable 16); glucose (%) (variable 17); D-glycero-D-mannoheptose (%) (variable 18); L-glycero-D-mannoheptose (%) (variable 19); myristic acid (%) (variable 20); β -hydroxymyristic acid (%) (variable 21); palmitoleic acid (%) (variable 22); palmitic acid (%) (variable 23). The data matrix of the measurements is not presented.

In this study we used two projection methods: PC and PLS (for references see Introduction). With both methods the original space for variable measurements is projected down onto two low-dimensional subspaces. One of these is sample-related, the other is variable-related. This projection also decides which of the variables contributes to the sample-related projection. The complexity of both models was determined by cross-validation (Wold 1976; Wold *et al.*, 1983). The basic principle of the SIMCA method is to model the data of a class of objects separately with a PC model. The complexity of the model was determined by cross-validation (Wold, 1978).

Results

The results of the individual studies providing the data sets of the present analyses have been described previously (Brondz & Olsen, 1984, 1985a, b, c; Olsen & Brondz, 1985). Therefore, only a brief summary will be given here.

Sugars and fatty acids

The most outstanding feature of gas-chromatographic analyses of whole-cell methanolysates was the absence of D-glycero-D-mannoheptose in *H. aphrophilus*. Representative strains of all the other species of the family

Pasteurellaceae examined contained this aldoheptose. The amount of D-glycero-D-mannoheptose present in *A. actinomycetemcomitans* and *H. paraphrophilus* was comparable, but was lower in *H. influenzae*. In *Pasteurella* spp., the amount of D-glycero-D-mannoheptose was highly variable. The contents of glucose and galactose were higher in *H. aphrophilus* than in *A. actinomycetemcomitans*. The glucose content of *H. paraphrophilus*, *H. influenzae* and *Pasteurella* spp. varied. The C_{14:0} content was similar in *A. actinomycetemcomitans*, *H. aphrophilus* and *Pasteurella* spp., but showed marked variation in *H. paraphrophilus* and *H. influenzae*. The C_{16:0} content varied considerably among the species examined.

Bacteriolysis

A. actinomycetemcomitans was more sensitive to bacteriolysis with EDTA or EDTA/HEWL than was *H. aphrophilus*. *A. actinomycetemcomitans* could be divided into two groups of strains according to differences in bacteriolysis: in group I, EDTA had a considerable lytic effect, which was not increased by supplementation with HEWL. In group II, the lytic effect of EDTA was much lower but HEWL had a considerable lytic effect. Maximal lysis of *A. actinomycetemcomitans* in the presence of EDTA occurred at pH 8.0, and with EDTA/HEWL at pH 7.6. *H. aphrophilus* exhibited maximal lysis by EDTA at pH 9.0, and by EDTA/HEWL at pH 9.2. When other members of the family Pasteurellaceae (*H. influenzae* type b, *H. paraphrophilus*, *P. multocida*, *P. haemolytica* and *P. ureae*) were included for comparison, the group I strains of *A. actinomycetemcomitans* were the most rapidly lysed by EDTA. *H. paraphrophilus* was the least EDTA-sensitive species tested, but not as resistant as *Micrococcus luteus* (control). *M. luteus* was the organism most sensitive to HEWL, followed by *P. ureae* and the group II strains of *A. actinomycetemcomitans*. The group I strains of *A. actinomycetemcomitans*, *H. paraphrophilus* and *P. haemolytica* were the least sensitive organisms.

Methylene blue reduction

In the *Actinobacillus-Haemophilus-Pasteurella* group, *P. haemolytica* and *P. ureae* reduced methylene blue most rapidly, followed by *P. multocida* and *H. influenzae* type b; the former completely reducing methylene blue within 5 min. The *H. aphrophilus* strains usually reduced methylene blue within 15 min, whilst the strains of *A. actinomycetemcomitans* were more variable.

PC analysis

The full data matrix of the measurements is available

Fig. 1. (a) PC score plot (t_2 vs t_1) based on data for samples 1–24 (Table 1). ○, *A. actinomycetemcomitans* (1–10); ●, *H. aphrophilus* (11–17); □, *H. paraphrophilus* (18–19); ■, *H. influenzae* (20–21); △ *Pasteurella* spp. (22–24). (b), Variable loading plot corresponding to the analysis in Fig. 1a. For variable numbering, see Methods. The first principal component describes 52% of the variance and the second component 11% of the variance in the data.

Fig. 2. (a) PLS discriminant analysis sample-related projection based on the classes *A. actinomycetemcomitans* (1–10) (○) and *H. aphrophilus* (11–17) (●). The remaining samples – *H. paraphrophilus* (18–19) (□), *H. influenzae* (20 and 21) (■) and *Pasteurella* spp. (22–24) (△) – are projected down onto the discriminating plane. (b) Variable loading plot corresponding to the analysis in Fig. 2(a). The first dimension describes 53% and the second 9% of the variance of the X -block.

from the authors. To obtain a general overview of the data, and without using the information available on species designation, a PC analysis was performed. The sample- and variable-related projections corresponding to the two first principal components are shown in Fig. 1(a, b). The first principal component described 52% of the variance and the second principal component 11% of the variance in the data. The sample-related projection showed that the first principal component along the x -axis distinguished *A. actinomycetemcomitans* (samples 1–10) from *Haemophilus* spp. (samples 11–21). The *Pasteurella* samples (22–24) fell close to those of *A. actinomycetemcomitans*. The variables that contributed most to this distinction were those with high absolute loading values for the first component, i. e. 1–14 and 16–17 (Fig. 1b).

PLS discriminant analysis

The first 17 samples in Table 1, classified previously as *A. actinomycetemcomitans* (samples 1–10) and *H. aphrophilus* (samples 11–17), were assumed to represent strains of these two species. To test this assumption a PLS discriminant analysis was performed. A two-dimensional ($A = 2$) plot was calculated for the 17 samples with both dimensions containing predictive class-separating information according to cross-validation. The first dimension described 53% and the second dimension 9% of the variance of the X -block. The remaining samples (18–23) were then projected down onto the sample-related plane (Fig. 2a). The corresponding variable-related projection is shown in Fig. 2(b). *A. actinomyce-*

Fig. 3. PLS discriminant plot based on the sugar variables. ○, *A. actinomycetemcomitans* (1–10); ●, *H. aphrophilus* (11–17).

Fig. 4. PLS discriminant plot based on the fatty acid variables. ○, *A. actinomycetemcomitans* (1–10); ●, *H. aphrophilus* (11–17).

temcomitans and *H. aphrophilus* classes (samples 1–10 and 11–17) were clearly dissimilar (Fig. 2a). The *H. paraphrophilus* (18 and 19) and *H. influenzae* samples (20 and 21) fell among those of *H. aphrophilus* in the plot. The *Pasteurella* samples (22–24) did not fit the discrimination plane well and fell between the *A. actinomycetemcomitans* and *H. aphrophilus* classes. From Fig. 2(a) it is clear that the first score vector, t_1 , distinguishes the *A. actinomycetemcomitans* class from the *H. aphrophilus* class. As can be seen from Fig. 2(b), a majority of the variables contributed class-discriminating information. The only exceptions were variables 15 and 19–23.

Predictive information provided by sugar and fatty acid variables

Two separate PLS analyses were made from the sugar and fatty acid variables derived from *A. actinomycetemcomitans* and *H. aphrophilus* strains. The four sugar variables could distinguish the two classes from each other (Fig. 3), whilst no class distinction was obtained with the fatty acid variables (Fig. 4). Cross-validation (Wold, 1976; Wold *et al.*, 1983) showed that the PLS model with the fatty acid variables contained no predictive information with respect to class discrimination, whilst for the model with the sugar variables the two latent vectors in Fig. 3 contained predictive information.

A. actinomycetemcomitans and *H. aphrophilus* class models (SIMCA analysis)

To establish borders for the *A. actinomycetemcomitans* class a separate PC model with $A = 3$ was calculated from the samples 1–10. The number of significant cross-terms of A was determined using cross-validation (Wold,

Fig. 5. Projection of the square root of the residual standard deviation for each sample fitted to the *A. actinomycetemcomitans* (1–10) class models ($\sqrt{s^{(1)}}$) and the *H. aphrophilus* (11–17) class model ($\sqrt{s^{(2)}}$). Dotted lines mark approximate class borders based on F -tests ($P = 0.05$). Samples 18 and 19, *H. paraphrophilus*; 20 and 21, *H. influenzae*; 22–24, *Pasteurella* spp.

1978). The additional samples 11–24 were adapted to this model. The residual variance of each object (11–24) was compared using an F -test to the pooled residual variances for samples 1–10. The residual variances for 1–10 were calculated by leaving out one sample at a time and by fitting the excluded sample to the reduced model with nine samples. None of the samples 11–24 fell within the 95% confidence limit of the *A. actinomycetemcomitans* class. The closest sample was no. 24, with an F -value of 3 compared to the critical value of 1.7.

A class model with $A = 2$ was determined for the *H. aphrophilus* class, and the same procedure as for *A.*

Fig. 6. PC score for the *Haemophilus* samples. ●, *H. aphrophilus* (11–17); □, *H. paraphrophilus* (18, 19 and 25); ■, *H. influenzae* (20 and 21). PC analysis was based on variables 16–23.

actinomycetemcomitans was used to establish borders for this class. None of the samples 1–10 or 18–24 fell within the 95% confidence limits of the *H. aphrophilus* class. The closest samples were 19–21 with *F*-values of 2.5, compared to the critical value of 1.7.

The results of the classification of the samples 1–24, when fitted to the two class models, are presented in Fig. 5. In this projection the approximate class borders are drawn.

Measurements for variables 1–15 were not available for sample 25, i.e. *H. paraphrophilus* ATCC 29242. A PC projection based on variables 16–23 and samples 11–21 and 25 (Fig. 6) shows the different behaviour of *H. paraphrophilus* samples (18, 19 and 25) in relation to that of *H. aphrophilus* samples (11–17) and *H. influenzae* samples (20 and 21).

Discussion

The multivariate analyses showed that *A. actinomycetemcomitans* (samples 1–10) and *H. aphrophilus* (samples 11–17) formed two dissimilar classes (Figs. 1a, 2a, 3 and 5). This was also demonstrated in studies based on high-resolution two-dimensional electrophoresis of cell proteins (Jellum *et al.*, 1984; Olsen *et al.*, 1987) and multilocus enzyme electrophoresis (Olsen & Caugant, 1988). The strains of *Pasteurella* spp. (samples 22–24) as well as those of *H. paraphrophilus* (samples 18 and 19) and *H. influenzae* (samples 20 and 21) were too few to be the basis for distinct class models. However, these samples fell outside both the *A. actinomycetemcomitans* and the *H. aphrophilus* classes. The *H. paraphrophilus* and *H. influenzae* samples fell just outside the border of the *H. aphrophilus* class (Fig. 5).

The analyses revealed that all variables except fatty

acids contained discriminatory information (Fig. 2b). No class distinction between *A. actinomycetemcomitans* and *H. aphrophilus* was obtained with the fatty acid data, in contrast to the discrimination obtained with the sugar variables (compare Figs 3 and 4). Cell sugars are therefore preferable to fatty acids as chemotaxonomic parameters for the *Actinobacillus*–*Haemophilus*–*Pasteurella* group.

For a data-analytical problem such as that presented here, where discrimination between classes is of interest, but where the number of variables are approximately 2–3 times the number of samples in the major classes, the risk for chance correlations are obvious if one variable at a time is studied, or if methods based on multiple regression are used (Topliss & Edwards, 1979). One cannot, therefore, by directly screening one variable at a time, conclude that the D-glycero-D-mannoheptose variable is a 'true' indicator variable despite the fact that it perfectly distinguishes the *A. actinomycetemcomitans* and *H. aphrophilus* classes. This distinction may be the result of a chance correlation. However, it appeared that the D-glycero-D-mannoheptose variable was not crucial for the discrimination of the *A. actinomycetemcomitans* and *H. aphrophilus* classes, since most of the other variables also contributed to class distinction. Using PLS and SIMCA we confirmed that the distinction of *A. actinomycetemcomitans* and *H. aphrophilus* was only marginally affected by exclusion of the D-glycero-D-mannoheptose variable. This supported the idea that the D-glycero-D-mannoheptose variable is, in fact, a 'true' indicator variable of *A. actinomycetemcomitans* and *H. aphrophilus*. Such variables can be distinguished from 'false' indicator variables, since a 'false' indicator variable may discriminate the classes from each other but the variable expresses variability within samples which is not reflected in other variables. The information from a 'true' indicator variable, however, is contained in numerous other variables. This information will appear significant in PC and PLS discriminant projection models. However, we are of the opinion that multivariate methods should not be used with the purpose of finding indicative variables.

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