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THE USE OF X-RAY PHOTOELECTRON SPECTROSCOPY FOR THE STUDY OF ORAL STREPTOCOCCAL CELL SURFACES

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Abstract—Physicochemical and structural properties of microbial cell surfaces play an important role in their adhesion to surfaces and are determined by the chemical composition of the outermost cell surface. Many traditional methods used to determine microbial cell wall composition require fractionation of the organisms and consequently do not yield information about the composition of the outermost cell surface. X-ray photoelectron spectroscopy (XPS) measures the elemental composition of the outermost cell surfaces of micro-organisms. The technique requires freeze-drying of the organisms, but, nevertheless, elemental surface concentration ratios of oral streptococcal cell surfaces with peritrichously arranged surface structures showed good relationships with physicochemical properties measured under physiological conditions, such as zeta potentials. Isoelectric points ap-peared to be governed by the relative abundance of oxygen- and nitrogen-containing groups on the cell surfaces. Also, the intrinsic microbial cell-surface hydrophobicity by water contact angles related to the cell-surface composition as by XPS and was highest for strains with an elevated isoelectric point. Inclusion of elemental surface compositions for tufted streptococcal strains caused deterioration of the relationships found. Interestingly, hierarchical cluster analysis on the basis of the elemental surface compositions revealed that, of 36 different streptococcal strains, only four *S. rattus* as well as nine *S. mitis* strains were located in distinct groups, well separated from the other streptococcal strains, which were all more or less mixed in one group.

Key words: X-ray photoelectron spectroscopy, oral streptococci, cell surface, physicochemical properties.

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The chemical composition of the outermost microbial cell surface determines adhesive cell-surface properties, such as charge (James, 1991) and hydrophobicity (Van Loosdrecht *et al.*, 1987; Mozes *et al.*. 1987) and therewith is an important factor in microbial adhesion to surfaces. Often, destructive methods are required for determination of the chemical composition of microbial cell walls which, in addition, do not yield the composition of the outermost cell surface (Bleiweis *et al.*, 1976; McBride *et al.*, 1984). X-ray photoelectron spectroscopy (XPS) is a well-known technique for the chemical characterization of solid surfaces such as, *e.g.*, polymers, metals, enamel, and dentin (Uchtmann and Duschner, 1982; Andrade, 1985; Kuboki *et al.*, 1987). The main advantages of XPS over many other techniques include its high surface sensitivity and its ability to provide reliable, quantitative chemical compositions.

The application of XPS as a tool for studying the chemical composition of microbial cell surfaces has, for a long time, been considered useless due to the complexity of microbial cell surfaces (Hammond *et al.*, 1984). Over the last decade, however, the pioneering work of Rouxhet and co-workers (Rouxhet and Genet, 1991) has stimulated the characterization of a wide variety of microbial strains and species, such as, *e.g.*, brewery yeasts (Amory *et al.*, 1988; Mozes *et al.*, 1988), oral bacteria (Van der Mei *et al.*, 1988a; Busscher *et al.*, 1991; Cowan *et al.*, 1992a,b), staphylococci (Van der Mei *et al.*, 1989; Denyer *et al.*, 1990), dairy strains (Van der Mei *et al.*, 1993), lactobacilli (Mozes and Lortal, 1995), isolates from urinary catheters (Jucker *et al.*, 1996), and *Escherichia coli* (Harkes *et al.*, 1993; Latrache *et al.*, 1994).

XPS is a high-vacuum (10^{-8}-10^{-9} torr) technique, requiring freeze-drying of the cells, which brings their cell surfaces into a state far remote from their physiological ones. Despite the fact that freeze-drying is used routinely in microbiology for long-term preservation of micro-organisms, the integrity of the outermost cell surface can be affected by the freeze-drying, exposure to the high vacuum, and X-ray irradiation (Marshall *et al.*, 1994). These types of problems are, however, anticipated more for Gram-negative than for Gram-positive strains, which have a relatively rigid cell wall. Also, like almost all physicochemical methods for the study of microbial cell-surface properties, the spatial resolution of XPS is inadequate to deal with chemical and structural heterogeneities, like sparsely or unevenly distributed fibrils or fimbriae on cell surfaces.

This review is confined to our XPS work on oral streptococci, both peritrichous and tufted, and aims to illustrate the relevance of XPS analyses of microbial cell

surfaces by describing relationships between elemental cell-surface compositions of 36 Gram-positive oral streptococci and isoelectric points and cell-surface hydrophobicity. In addition, we investigated whether a grouping of the strains could be obtained by hierarchical cluster analysis based on the physicochemical cell-surface properties measured that correspond with their taxonomy.

MATERIALS AND METHODS

Growth conditions

The 36 oral streptococcal strains included this review are listed in the Table. A detailed description of their cell-surface characteristics is given in the references indicated. For each experiment, the bacteria were grown overnight at 37°C from a frozen stock in batch culture in Todd-Hewitt broth. This culture was used to inoculate a second culture, which was grown for 16 hrs and then harvested by centrifugation for 5 min at 5000 *g*, washed twice with demineralized water, and finally suspended in appropriate solutions for zeta potential measurements, in demineralized water for contact angle measurement or freeze-dried for XPS.

TABLE

ORAL STREPTOCOCCAL STRAINS INCLUDED IN THIS REVIEW AND REFERENCES GIVING DETAILS ON THEIR PHYSICOCHEMICAL CELL-SURFACE PROPERTIES

# of Oral Streptococcal Strains *per* Species	References
5 *S. salivarius*	Van der Mei *et al.*, 1988b
6 *S. sanguis*	Busscher *et al.*, 1991
	Van der Mei *et al.*, 1988a
6 *S. mutans*	Van der Mei *et al.*, 1988a
	Van der Mei *et al.*, 1991
3 *S. sobrinus*	Van der Mei *et al.*, 1991
3 *S. cricetus*	Van der Mei *et al.*, 1991
4 *S. rattus*	Van der Mei et al., 1991
9 *S. mitis*	Cowan *et al.*, 1992a

Particulate microelectrophoresis

The zeta potentials of the streptococci were measured as previously described (Van der Mel *et al.*, 1988b). Briefly, each bacterial strain was suspended in 50 mL of 10 mM potassium phosphate solution, set at a pH of 2, 3, 4, 5, 7, or 9 by means of either HCl or KOH, to a concentration of approximately 1 x 10^7 cells/mL. The electrophoretic mobility at 150 V of the suspended bacteria was then measured by means of a Lazer Zee Meter 501 (PenKem, Bedford Hills, NY, USA), which uses the scattering of incident laser light to detect the bacteria. The mobility of the bacteria under the applied voltage was converted to the zeta potential according to the Smoluchowski equation (Hiemenz, 1977). Isoelectric points (IEP), *i.e.*, the pH at which positive and negative charges counterbalance each other, were determined from plots of the zeta potentials *vs.* pH, either by interpolation or careful extrapolation.

Contact angle measurement

Contact angle measurements were deter-mined by means of a technique originally developed by Van Oss and Gillman (1972). Briefly, each bacterial strain was layered onto a 0.45-µm-pore-size filter (Millipore, Bedford, MA, USA) by means of negative pressure. The filters were left to air dry at room temperature and humidity until so-called plateau water contact angles could be measured (approximately 1 hr). For each strain, three independently grown cultures were used, from which two filters of each were prepared and measured. The contact angles, made by sessile droplets of water on the bacterial layers, were measured means of an automated contour monitor and taken as a measure for the intrinsic cell-surface hydrophobicity.

X-ray photoelectron spectroscopy (XPS)

The washed bacterial pellets were transferred to stainless steel troughs, frozen in liquid nitrogen, and subsequently freeze-dried in a Lyovac GT4 or Combitron CM30 (Leybold Heraeus, Cologne, Germany) freeze-drier. The freeze-dried samples were pressed into small stainless steel cups, and put into the XPS chamber (either a VG-ESCA 3 Mk II, Sussex, UK, or an S-Probe, Surface Science Instruments, Mountain View, CA, USA). X-ray production occurred by means of a magnesium anode. Scans were made of the overall spectrum in the binding energy range of 1-1200 eV at low resolution, then peaks over a 20-eV binding energy range were recorded at high resolution for C_{1s}, O_{1s}, N_{1s}, and P_{2p}. The area under each peak, after background subtraction, was used for the calculation of peak intensities, yielding elemental surface concentration ratios for nitrogen, oxygen, and phosphorus to carbon, after correction with sensitivity factors appropriate to the instrument used.

Statistical analysis

Statistical analyses were done with the SPSS/PC+ statistical program package. The water contact angles were used as measured in degrees, while the N/C, O/C, and P/C elemental surface concentration ratios and the isoelectric points were multiplied by 500, 100, 5000, and 10, respectively, so that they would be on an absolute scale similar to that for the water contact angles.

A hierarchical cluster analysis based on squared Euclidean distances was carried out based on all cell-surface properties measured and based solely on the XPS data as input. The output of the cluster analysis was presented in the form of dendrograms in which strains possessing a great similarity are combined at small distances, and unlike strains remain separated up to larger distances (ten Bosch *et al.*, 1991).

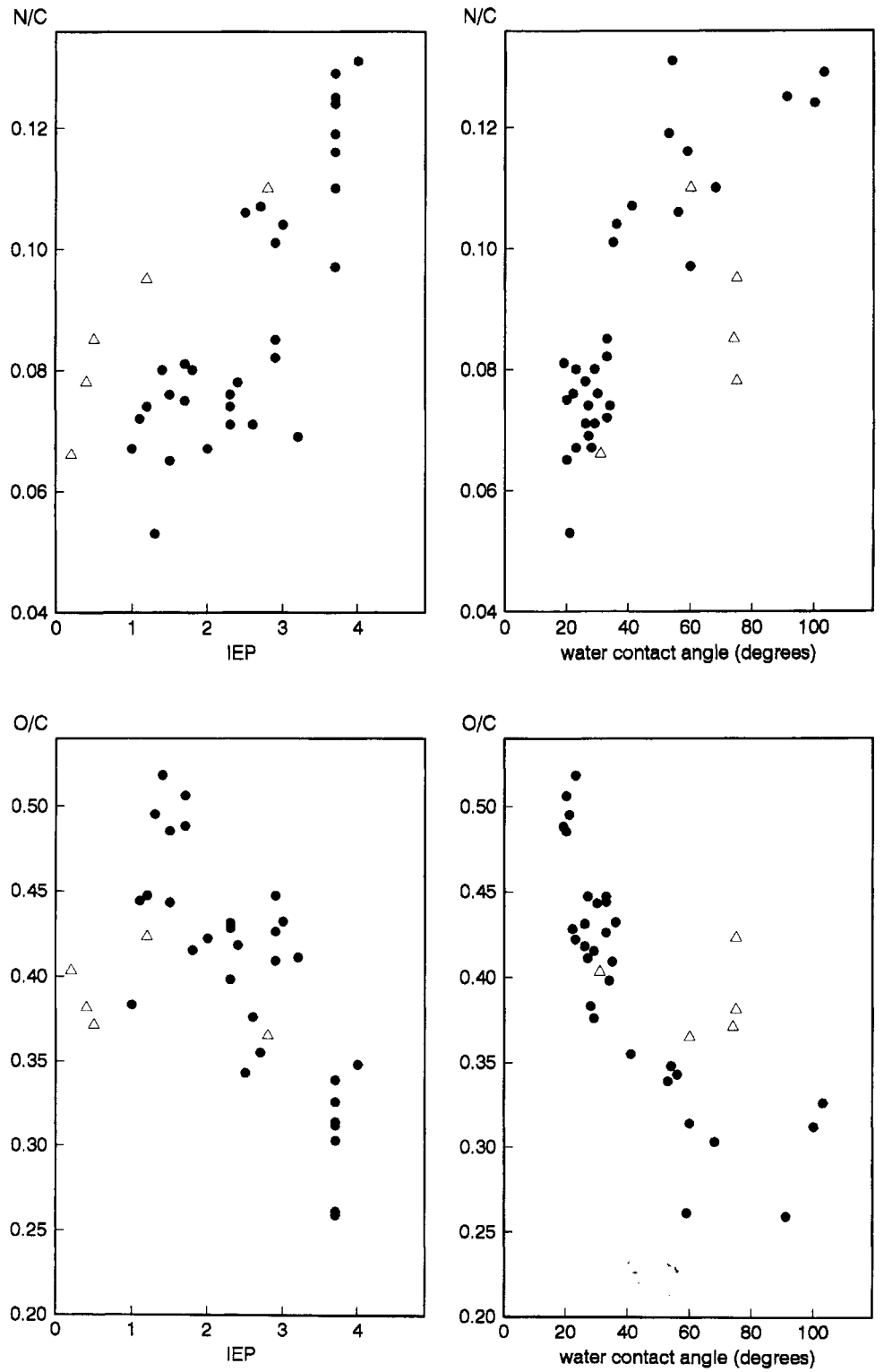

Fig. 1—Elemental surface concentration ratios N/C and O/C as by XPS of peritrichous oral streptococci (•) and of tufted streptococci (Δ) as a function of their isoelectric points in 10 mM potassium phosphate solutions and their intrinsic cell-surface hydrophobicity by water contact angles.

RESULTS

The elemental surface compositions N/C and O/C measured on freeze-dried oral streptococci by XPS are plotted *vs.* their isoelectric points and intrinsic cell-surface hydrophobicities as measured by water contact angles (Fig. 1). All other details of their physico-chemical cell-surface properties, including their P/C elemental surface concentration ratios, can be found in the references given in the Table. In Fig. 1 it can be seen that the water contact angles of the streptococci range from 19 to 103 degrees, *i.e.*, from strongly hydrophilic (mostly the *S. rattus* strains) to strongly hydrophobic (predominantly the *S. mitis* strains). Also, the streptococcal isoelectric points can vary widely from 0.2 to 4. Relationships between the N/C and O/C elemental surface concentration ratios and the isoelectric points and water contact angles become obvious from Fig. 1 as well, with a positive correlation between the N/C elemental surface concentration ratio and the isoelectric points and water contact angles. Possibly, this indicates that nitrogen-rich components, most notably proteins, convey hydrophobicity to the oral streptococcal cell surfaces, thereby simultaneously increasing their isoelectric points. A negative correlation between the O/C elemental surface concentration ratio and the isoelectric points and water contact angles was found, which may point to the fact that oxygen-rich components, as phosphates and polysacccharides, convey negative charge and hydrophilicity to the cell surfaces.

Interestingly, the tufted *S. sanguis* strains roughly follow the types of relationships found for all other peritrichous streptococci, but still are always somewhat off the general relationships. A hierarchical cluster analysis based solely on the XPS data yields a

grouping of the *S. mitis* and *S. rattus* strains (see Fig. 2), presumably as a cause of their relatively high N/C and O/C elemental concentration ratios, respectively. When the isoelectric points and water contact angles are included in the cluster analysis, the *S. rattus* strains remain grouped in one separated cluster, but the cluster comprised of the *S. mitis* strains is divided into two reasonably well-separated clusters by the tufted *S. sanguis* strains (see Fig. 3).

DISCUSSION

In this review, it is shown that the elemental surface compositions of oral streptococcal cell surfaces as determined by XPS show excellent relationships with isoelectric points and water contact angles, especially when confined to peritrichous strains. Although XPS requires freeze-drying of the micro-organisms and is carried out in high vacuum under X-ray irradiation, the relationships observed with isoelectric points measured by particulate microelectrophoresis with the cells in physiological circumstances are taken as evidence in support of XPS as a valuable tool for the study of microbial cell surfaces. Similar evidence can be taken from work on other Gram-positive micro-organisms, like

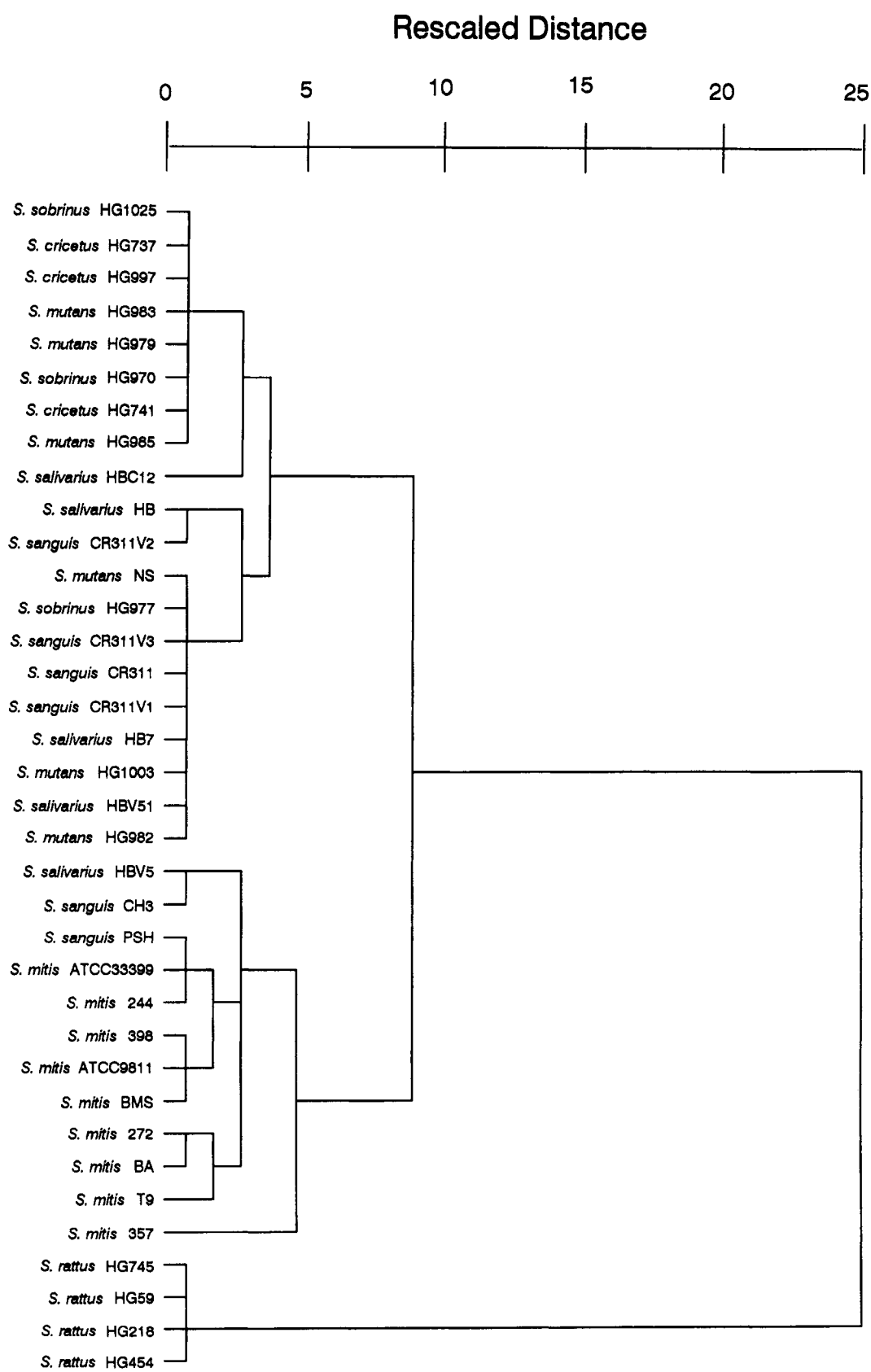

Fig. 2—Dendrogram resulting from a hierarchical cluster analysis based on the elemental surface compositions of oral streptococci, as determined by XPS.

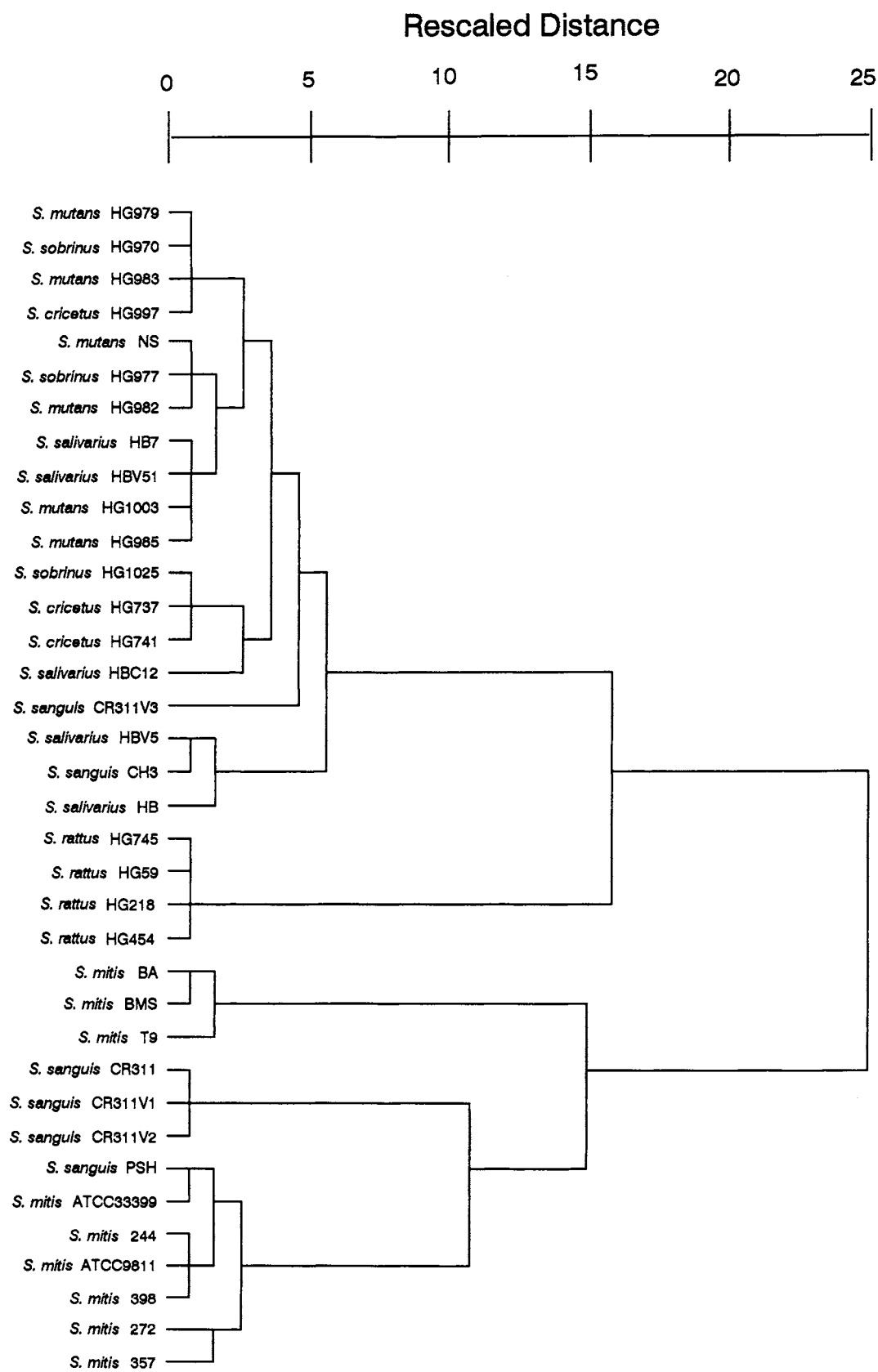

Fig. 3—Dendrogram resulting from a hierarchical cluster analysis based on the elemental surface compositions of oral streptococci, as determined by XPS and on their isoelectric points and cell-surface hydrophobicities.

lactobacilli (Cuperus *et al.*, 1993; Millsap *et al.*, 1997). Generally, the reproducibility of XPS analyses can be classified as good, and duplicate experiments usually correspond within 10% or better (Rouxhet *et al.*, 1994). Comparison of analyses taken over a seven-year time span with two different freeze-driers and XPS instruments on *S. salivarius* HB yielded N/C and O/C elemental surface concentration ratios of 0.104 and 0.432, respectively (Van der Mei *et al.*, 1988b) and of 0.105 and 0.456, respectively (1995; unpublished).

Nevertheless, caution should be exercised when applying XPS to Gram-negative microbial strains, as was correctly pointed out by Marshall *et al.* (1994). Until now, no convincing relationships have been described for Gram-negative strains between XPS data and other physicochemical cell-surface properties, and in studies on Gram-negative *Escherichia coli* and Gram-negative subgingival bacteria, no relationships were observed at all (Cowan *et al.*, 1992b; Harkes *et al.*, 1993). Presently, it is not known whether this is because of damage done to the fragile cell surfaces of Gram-negative microorganisms during sample preparation or because of the greater complexity of the Gram-negative cell sur-

face (Hammond *et al.*, 1984; Marshall *et al.*, 1994).

Caution should also be exercised in the study of strains that are not peritrichous. The tufted *S. sanguis* strains, denoted by the triangles in Fig. 1, have their fibrils arranged in tufts on the lateral equator of the cell surface (Busscher *et al.*, 1991), in contrast to the other strains used, which are either bald or have their fibrils peritrichously spread over the whole cell surface (Van der Mei *et al.*, 1988a,b; Cowan *et al.*, 1992a). It is easy to envisage how a macroscopic technique like XPS could over- or underestimate the overall chemical composition of a non-uniform cell surface. Similarly, it cannot be ascertained in any easy way whether the zeta potentials or cell-surface hydrophobicities are determined in a non-proportional way by the tufts, as might be inferred from the observation that inclusion of data for the tufted *S. sanguis* strains in Fig. 1 yields a deterioration of the relationships shown.

It is difficult, if not in principle impossible, to obtain a grouping of microbial strains on the basis of their cell-surface properties in accordance with their taxonomy, unless strains and species possess distinctly different surface compositions. In this study, the *S. rattus* and the *S. mitis* strains distinguish themselves by a high amount of cell-surface oxygen and nitrogen, respectively. For the *S. rattus* strains, the high amount of surface oxygen is accompanied by the absence of an isoelectric point over the entire pH range from 2 to 9, while for the *S. mitis* strains, the high amount of surface nitrogen causes the elevated intrinsic cell-surface hydrophobicity of the strains. Although an identical study (Millsap *et al.*, 1997), as described here, on 27 lactobacillus strains, encompassing 4 species, grouped *Lactobacillus acidophilus* in a cluster that was well-separated from the other lactobacillus species, strains of other species were not grouped together. In this respect, therefore, it must be concluded that, in general, the phenotypic appearance of a bacterium in terms of its physicochemical cell-surface properties cannot be related to the microbial taxonomy.

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