SURFACE ANALYSIS AND DEPTH PROFILE COMPOSITION OF BACTERIAL CELLS BY X-RAY PHOTOELECTRON SPECTROSCOPY AND OXYGEN PLASMA ETCHING

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SUMMARY. X-ray photoelectron spectroscopy (XPS) was used to analyze the outermost (2-5 nm) surface of bacterial cells. Elemental analysis of the cell surfaces in Bacillus subtilis 168 and Bacillus megaterium KM gave a strong P signal attributed to teichoic acids. Teichoic acid-less Micrococcus lysodeikticus ha a very weak P signal. Oxygen plasma etching (OPE) combined with XPS and electron microscopy was used to obtain depth profiles of the cell surfaces. Distribution of P (teichoic acid) throughout the cell wall of the two Bacillus species was demonstrated. Separation of the two membranes in Escherichia coli B by their P signal was however not achieved. Na, the common surface cation, was replaced by K upon surface etching. Atomic ratios (C:O:N) of the surface biopolymers essentially agreed with known surface composition.

X-ray photoelectron spectroscopy (ESCA, XPS) has been used to analyze the surface of solid materials (1,2). XPS gives the energy spectra of electrons that are photoejected by exciting X-rays from the surface layers of a specimen (3-7). The spectra provide information about binding energy of the ejected electrons and therefore the elemental composition of the specimen surface. The surface depth so analyzed is on the order of a few nanometers (3). Low temperature oxygen plasma etching oxidizes the surface layers of a specimen at temperatures below 100^0, etching away the organic matrix leaving behind mineral oxides and salts of the exposed elements (8,9). The morphological results of etching can be ascertained by electron microscopy (8-10).

A combination of surface analysis obtained by XPS and oxygen plasma treatment of biological specimens should therefore not only yield the elemental composition of the outermost surface but also in-depth elemental profiles of the same specimens as etching progresses. This rationale was applied to the study of the distribution of teichoic acids in the cell walls of Gram-positive bacteria , the nature of cations on the cell surfaces, the layering of the envelopes in Gram-negative bacteria and the macromolecular composition of the cell surfaces.

Reference to a company and/or product named by the Department is only for purposes of information and does not imply approval or recommendation of the product to the exclusion of others which may also be suitable.

MATERIALS AND METHODS

Microorganisms. Bacillus subtilis 168, Bacillus megaterium KM, Micrococcus lysodeikticus and Escherichia coli B were grown in aerated Bacto-Antibiotic medium # 3 (Difco) (11) at 35° for 12 hrs. Cells were harvested by centrifugation and washed in cold distilled water. Cell walls of B.subtilis 168 were obtained by sonicating aqueous cell suspensions in a Raytheon 10 kC sonic oscillator. The walls were cleaned by differential centrifugation and washed in dist.water.

Specimen supports. Disks (6 mm diameter) were cut from either a 0.5 mm thick sheet of polystyrene-polybutadiene plastic, "Resin K" (Phillips Petroleum) or from microscope cover glass slips no. 1 1/2 (Corning). For XPS, the glass disks were usually coated with SiO to a thickness of about 35 nm in a vacuum evaporator (8). For electron microscopy, 200-mesh stainless steel grids were filmed with collodion and similarly coated with SiO (8). Immediately before use, all specimen supports were rendered hydrophilic by a brief oxygen plasma treatment (9).

Specimen preparations. Ten microliters of cell or wall suspension were deposited on the surface of a support disk and allowed to air dry at 25°. The suspension concentration which would give the best quasi-monocellular layer on the disk surface had been determined beforehand by phase contrast light microscopy (500 X magnification) of the transparent disks. For electron microscopy, specimen preparation was similar except that the droplet placed on a grid was largely withdrawn after 1 min and only a thin film allowed to air dry. Polystyrene latex spheres (264 + 6 nm diameter; lot LS-057-A , Dow Chemical) were usually sprayed onto the grids before application of the specimen. Grids were shadowed with uranium.

Plasma etching. The specimen surface was oxidized by exposure to reactive oxygen generated at room temperature in a plasma etcher, "Plasmod" (Tegal Corp.) (12). The plasma reactor was operated at its minimum power of less than 5 watts and with a continuous oxygen flow at a pressure of 1 torr.

Electron microscopy. A Hitachi HU-10 transmission electron microscope was used at 50 kV.

Photoelectron spectroscopy. Core electron binding energies were measured with an E.I.Du Pont de Nemours model 650 B electron spectrometer (magnesium anode) equipped with a multichannel analyzer. The X-ray source was used at its full power. The Au_{4f} elemental lines of a gold-coated aluminum foil were used as an external standard. Survey scans (wide energy scans of binding energy 550-50 eV) were done using 512 channels of the multichannel analyzer at a scan rate of 0.1 sec/channel. Data were accumulated over 16 scans. Single electron lines were obtained using a routine scan rate of 0.2 eV/sec. Line intensities were estimated by measuring peak heights or peak areas. Intensities were normalized using sensitivity factors (13) or cross-sections (14). The spectrometer was kept at a vacuum of 4×10^{-7} torr.

Disk coverage. The percentage decrease of the Si signal intensity of the SiO-coated support disk covered with the biological specimen as compared with the uncovered support disk was used as an indicator of disk (substrate) coverage(15).

RESULTS

Surface analysis of cells. Survey scans of the bacterial specimens indicated in a qualitative manner the essential features of the elemental spectrum (Fig.1). As expected, elemental lines of C_{1s}, N_{1s} and O_{1s} were the strongest. Elemental lines of P could be detected in all four bacterial species, although in vastly different amounts (Fig.2 and Table I). Na, detected mostly as the Auger KLL signal, was the common cation found at the cell surfaces (Fig.1 and 3). K was found only in small amounts and then only at the highest cell concentrations. No K was found in the isolated cell walls of B.subtilis which showed however a very strong Na signal giving a Na:N ration of 0.12:1 . An analysis

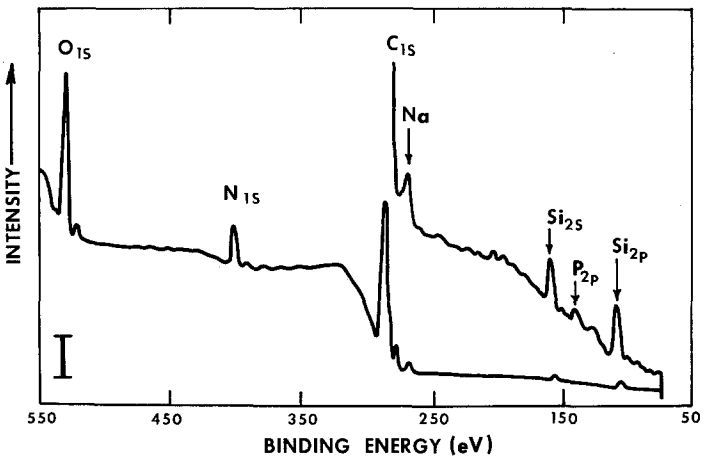

Fig. 1 . Survey scan of B.megaterium KM cells on SiO/glass disk. Na is Auger KLL line of Na. Bar = 1000 counts/sec (lower scan) or 125 counts/sec (upper)

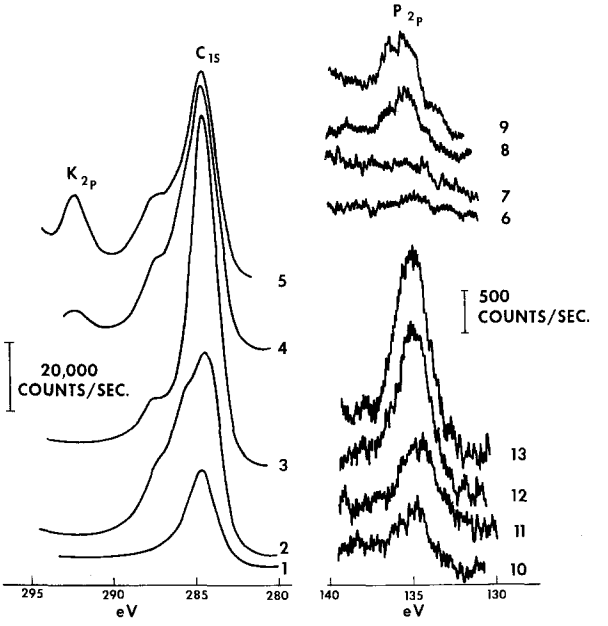

Fig. 2. Elemental line spectra. #1-5: C_{1s} and K_{2p} lines of SiO/glass (#1), B.subtilis 168 cell walls (#2), E.coli B cells (#3), M.lysodeikticus cells (#4), and M.lysodeikticus cells etched for 4 min (#5). #6-9: P_{2p} line of E.coli cells (#6), M.lysodeikticus cells (#7), B.megaterium (#8) and B.subtilis (#9) cells. #10-13: Etch series of the P_{2p} line of B.megaterium cells (sequential etching of the same specimen for 0, 2, 4 and 6 min.)

Table I. Line Intensity and Atom Ratio of Surface Elements of Bacteria as Obtained by XPS.

SPECIMEN expt. no.		specimen support	disk coverage	LINE INTENSITY[a] Si_{2p}	C_{1s}[b]	O_{1s}	N_{1s}	P_{2p}	$Au_{4f/2}$[c]	ATOM RATIO[d] N	C	O	P	Si
			%	10^3 counts/sec										
I	none	Resin K	-	0	41.6	2	0	0	100	0	13.4	1	0	0
V	none	SiO/glass	0	25	25.2	165	0	0	190	0	0.9[e]	2.2[e]	0	1[e]
II	growth medium	SiO/glass	100	-	-	-	-	-	125	1[e]	6.5[e]	8.7[e]	0.2[e]	0[e]
CELLS:														
V	E.coli B	SiO/glass	79	5.4	64.5	79	13.9	1	190	1	9.3	2.7	0.07	-
I	E.coli B	Resin K	-	-	24.6	-	-	-	100	1[e]	-	2.3[e]	-	-
V	M.lysodeikticus	SiO/glass	96	0.9	36	63.5	15	0.2	190	1	6.4	2.0	0.005	-
V	B.megaterium KM	SiO/glass	93	1.6	56	57	11.5	0.6	190	1	13.3	3.7	0.05	-
I	B.megaterium KM	Resin K	-	-	-	20.3	4.8	0.4	100	1	-	3.6	0.08	-
I	B.subtilis 168	Resin K	-	-	24	14.9	3.1	0.6	100	1	-	3.7	0.21	-
CELL WALLS:														
V	B.subtilis 168	SiO/glass	94	2.4	52	83.5	7.2	3.2	190	1	17.5	7.5	0.41	-
VII	N-acetyl-glucosamine	bulk specimen[f]		-	8.8	19.6	2.3	-	-	1	9.6	6.6	-	-

[a] Single element scan, not corrected for disk coverage or variations in Au standard counts. [b] C_{1s} peak height measured at 284-285 eV. [c] External (machine) gold standard. [d] Ratios based upon peak areas, corrected for disk coverage, except as indicated (footnotes e). [e] Ratios based on peak heights estimated from multiple scan spectra. [f] Compressed pellet.

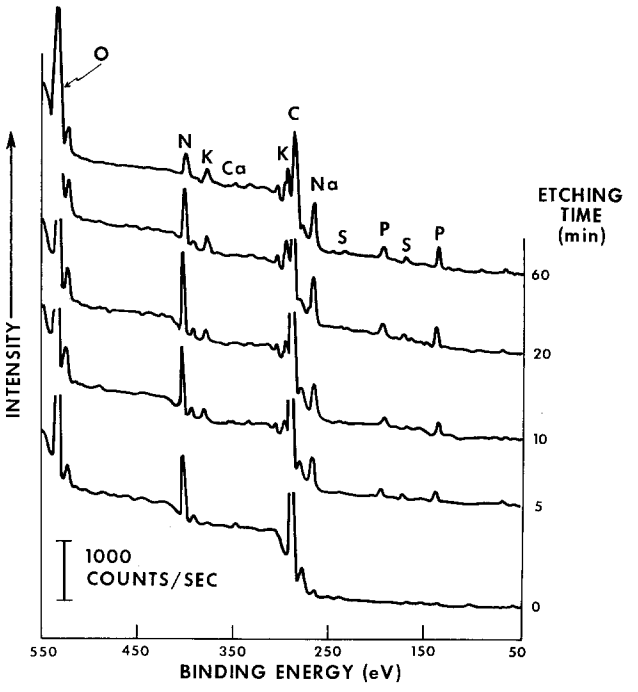

Fig. 3. Survey scans of E.coli B cells on Resin K support disk after different etching times (sequential etching of the same specimen).

of the line intensities and the atom ratios to N of C, O and P indicated significant differences between the four species (Table I). For a given species, however, the O:N and P:N ratios were not appreciably affected by variations in substrate (Resin K or SiO/glass) or substrate coverage. Whereas the O:N atom ratio of a reference compound, N-acetyl-glucosamine (computed ratio C:O:N of 8:6:1) or the O:Si ratio of the hydrophilized SiO-covered glass (SiO oxidized to SiO_2) were quite close to the theoretical expectations, C content was generally higher than expected. The surface analysis of the two Bacillus species gave essentially the same O:N atom ratio. P content varied widely, e.g. see P:N for M.lysodeikticus and B.subtilis cells in Table I. P is found in the inner and outer membranes of E.coli envelopes and in the teichoic acids of the Gram-positive Bacillus cell wall (16,17). M.lysodeikticus cell walls, on the other hand, contain essentially no P (18). The P:N ratios substantiate this (Table I).

Depth profile of E.coli cells. A series of wide scans showed changes produced by oxygen plasma etching on E.coli B cells deposited on Resin K (Fig.3). Etching added the elemental lines of P, S, K, Na and Ca to the already present ones of C, N and O. This particular sample exhibited a very low signal for P on the unetched surface , probably because of low disk coverage. During etching,

the signal intensities of N and C decreased (Fig.3). Increase of P signals from the etched surfaces appeared greatest during the first few minutes of etching (Fig.4). This may reflect true in-depth variations in the concentrations of these elements . This might also result, however, from initially higher etching rates, or eventual surface saturation with accumulated ash.

Oxygen plasma etching and electron microscopy of polystyrene latex spheres. As published etching rates vary considerably, and depend probably on instrumental conditions (19), it was felt that latex spheres of known diameter added to the biological samples and sized by electron microscopy after etching would provide an internal indicator of etching dose in microscopy experiments and also determine the magnitude and probable reproducibility of etching rates for the XPS experiments. Oxygen plasma etching uniformely decreased the initial diameter of the spheres (264 nm) at an average rate of 9.0 nm/min in an apparently linear fashion , at least during the first 10 min (10). Biological specimens undoubtedly etch at a different rate, but the rates are presumably related by some reproducible factor.

Depth profile and electron microscopy of Bacillus cells. As seen in Fig.2 and 4, the etching pattern, exemplified by an increase in P signal , followed the same trend as that seen in E.coli : steep initial increase during the first few minutes, and subsequent plateau formation. The Si signal increase, although slight, was an indication of probable cell shrinkage of B.megaterium (Fig.4)

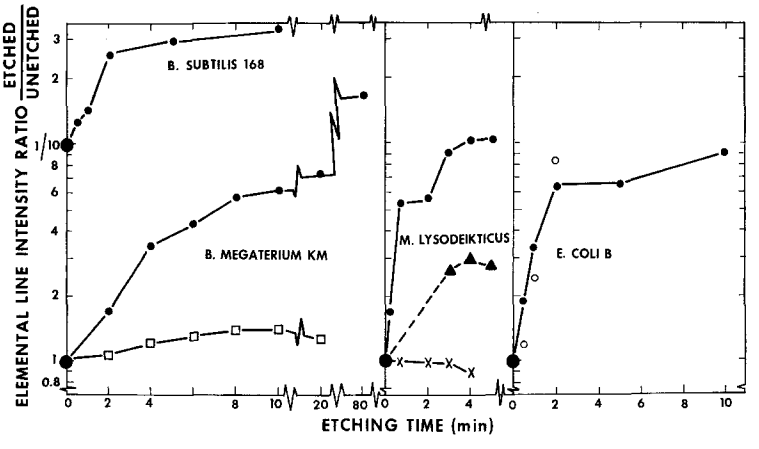

Fig. 4. Influence of the sequential etching time on the signal intensity of elements. Specimen supports: Resin K (B.subtilis), SiO/glass (others). ● - - ● P_{2p} ,□- -□Si_{2p} ,▲- -▲K_{2p} , X - - X N_{1s} . o - - o same as ● - - ● but single etching of different specimens.

confirmed by electron microscopy (10). Electron microscopic analysis of the etched cells or cell walls inidcated that after 30 sec etching, the isolated walls appeared thinner but both they and the intact cells still showed a smooth surface (10). One min etching gave a pitted aspect to isolated walls. Isolated walls had disappeared completely after 2 min etching. Etching for 5 min or longer clearly removed the walls from intact cells and selectively attacked interior structures (e.g. polyhydroxbutyrate granules). At extended etching times, one or two hours, an ash pattern probably devoid of organic material was produced, as seen by electron microscopy.

Depth profile of M. lysodeikticus. Etching also increased the P signal of this organism with a thick cell wall. A drastic increase of the K signal was also observed.

DISCUSSION

Escape depth of electrons. XPS theory indicates a mean (Gaussian) escape depth of electrons in polymers on the order of 2 nm (2,3). This means that 90 percent of the electrons analyzed come from a specimen depth of less than 5 nm (5). Partial coverage of the disks and unevenness of biological samples com - plicate without a doubt the production and spatial distribution of the ejected electrons. Presence of some cations like K at the unetched cell surface could indicate that the signal analyzed by XPS derived partially from the cell interior , K being usually considered to be found in the cell interior only. K might also leak out of the cells during air drying. Grazing angle experiments could clarify this point (15).

Contamination and radiation damage. C contamination which was responsible for the high C content of the atomic ratios, was one of the constant problems in all experiments. On the other hand, irradiation of the specimens by X-rays for 30 min did not appreciably decrease the P signal.

Oxygen plasma etching. The correlation of morphological alterations seen by electron microscopy with XPS signal intensity increase appears to indicate that during the first one or two minutes, etching uniformly volatilizes the organic matrix of the gram-positive cell wall and exposes subsurface inorganic constituents. Evidently, the etching at these short times can be used as a true depth profiling method, even though exact interpretation of the profile data requires furhter study. At longer etching times, the accumulation of ash residues which perhaps saturate the surface and the concommitant lack of XPS signal increase suggests that the requirements for a true depth profiling are no longer satisfied.

Distribution of teichoic acids in Gram-positive cell walls. As indicated above, oxygen plasma etching rates appeared to be constant during the first few min-

utes of etching. The thickness of the Bacillus cell wall has been estimated to be approx. 25 nm (see e.g. ref.20). As the P signal increases continuously during the time required to etch away the cell wall, it seems likely that teichoic acids (which produce the P signal in the cell wall) are distributed throughout the cell wall. The P signal in the teichoic-acid-less M.lysodeikticus can be attributed to traces of phosphoglucuronic acid (18) or the underlying cell membrane.

Gram-negative cell envelope. The methods used, XPS alone or in combination with plasma etching , apparently was not able to separate P of the inner and outer membrane of E.coli.

Cell surface cations. Although traces of K were usually found on the cell surface, the common outside cation was Na. A drastic increase in K signal during etching of M.lysodeikticus cells (Fig.4) appears to occur later than the corresponding P increase .

Atomic ratios of surface biopolymers. Elemental analyses of proteins, polysaccharides, aminosugars or lipids give different C:O:N ratios. Analysis of the data in Table I indicates that the Bacillus surfaces contain compounds rich in O , which agrees with the presence of carbohydrates or peptidoglycans with no interpeptide bridges (predominant role of the aminosugars) (21). M.lysodeikticus which has in its wall peptidoglycan longer interpeptide bridges (21), shows a lower N:O ratio. The presence of protein surface subunits, although never shown for the above mentioned species, might complicate the picture. Atomic ratios of the E.coli surfaces, with a ration N:C:O of 1:9.3:2.5 would indicate the predominance of proteins although the C content appears to be high for proteins.

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