

X-Ray Photoelectron Spectroscopy Analysis of Whole Cells and Isolated Cell Walls of Gram-Positive Bacteria: Comparison with Biochemical Analysis

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The surface chemical composition of whole cells and isolated cell walls of four coryneform bacteria and of a *Bacillus brevis* strain has been determined by X-ray photoelectron spectroscopy (XPS). The XPS data were converted into concentrations of model compounds: peptides, polysaccharides, and hydrocarbonlike compounds. The composition of the surface of *B. brevis* differed markedly from that of coryneforms: the peptide concentration was about twice higher in the former case, which is attributed to the presence of an S-layer at the cell surface; in contrast, the surface of coryneforms was rich in hydrocarbonlike compounds (about 40%), which was concomitant with a high water contact angle. The peptide surface concentration of the isolated cell walls of the five strains deduced from XPS data fitted well with the total peptide content determined by biochemical analysis, which supports the validity of XPS to determine the overall macromolecular composition of the bacterial cell surface. Compared to biochemical analysis of isolated cell walls, XPS analysis of whole cells provides information which concerns directly the cell surface (2- to 5-nm-thick layer) and is less subject to alteration via losses of cell wall constituents or contamination by intracellular compounds.

X-ray photoelectron spectroscopy (XPS) provides a direct chemical analysis of solid surfaces. The technique involves irradiation of the sample by an X-ray beam, which induces ejection of photoelectrons. The kinetic energy of the emitted electrons is analyzed, and their binding energy in the atom of origin is determined. Due to inelastic scattering of electrons in the sample, the collected information concerns only the outermost molecular layers of the surface (2 to 5 nm). Each peak of the recorded spectrum is characteristic of a given electron energy level of a given element, and its position is influenced by the chemical environment. Therefore, XPS provides an elemental analysis and a rough functional group analysis of the surface. Detailed information on the technique can be found in the literature (16, 17).

Although XPS is now well established in the surface study of inert materials, its use for the characterization of microbial cell surfaces is not yet widespread (1, 5, 6, 7, 8, 18, 21). Since the analysis is performed under high vacuum, the cells must be freeze-dried before being introduced in the spectrometer. This raises questions concerning the representativity of the analyzed surface with respect to the native surface in the hydrated state (10). The relevance of XPS to the probing of microbial surfaces has been supported by correlations between the XPS results and cell surface properties, such as hydrophobicity and electrical properties, and by relationships with the cell behavior at interfaces (1, 3, 5, 6, 8, 13, 18). However, attempts to compare directly the surface composition provided by XPS with the cell wall composition obtained by classical biochemical analysis are scarce (12).

Recently, the chemical composition of the cell walls of five gram-positive bacterial strains, including four coryneforms and

a *Bacillus brevis* strain, has been determined (22). For coryneform bacteria, peptidoglycan is an important cell wall component and contributes about 23 to 31% to the cell wall dry weight; the protein content—assessed from amino acid analysis—is in the range of 7 to 14%. The peptidoglycan fraction of *B. brevis* is much smaller (about 5%), whereas the amount of proteins is very large (>56%), most likely due to the presence of an S-layer.

In the present paper, the surface chemical composition determined by XPS is presented for whole cells and isolated cell walls of these five gram-positive bacterial strains. The surface composition is related to the surface hydrophobicity and compared with the cell wall composition obtained by biochemical analysis.

MATERIALS AND METHODS

Cell culture. The following strains have been examined: *Corynebacterium* sp. strain DSM 44016, *Corynebacterium* sp. strain DSM 6688, *Rhodococcus erythropolis* A177, *Rhodococcus opacus* C125, *B. brevis* ATCC 9999, *Corynebacterium* strains were cultivated in brain heart infusion broth (Merck, 40 g per liter of deionized water). *Rhodococcus* strains were cultivated in the medium described by Schraa et al. (19), except that yeast extract was not added; ethanol (50 mM) was used as the sole carbon and energy source. *B. brevis* ATCC 9999 was cultivated in (grams per liter): NH_4Cl (2.2), KH_2PO_4 (0.27), KNO_3 (0.05), $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (0.2), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (0.06), $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (0.01), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.35), Casamino Acids (7.5), and glycerol (2.5). Trace elements (1 ml per liter) and vitamin solution (1 ml per liter) were added (19). The bacterial cells were grown at 30°C on a rotary shaker and harvested in the early stationary phase by centrifugation for 10 min at $20,000 \times g$ at 4°C. The cells were washed three times in deionized water.

Cell wall isolation (22). A French pressure cell was used to disrupt the cells of the coryneforms at a pressure of $20,000 \text{ lb/in}^2$ and those of *B. brevis* at $15,000 \text{ lb/in}^2$. Guanidinium hydrochloride (4 M) was added in order to inactivate autolytic enzymes and to remove the cytoplasmic membrane. The disrupted cells were centrifuged at $30,000 \times g$, and the pellet was resuspended in 4 M guanidinium HCl. If necessary, the French press procedure was repeated once more. The broken cells were washed several times in 1 M KNO_3 and centrifuged at $1,500 \times g$ to remove intact cells and coarse debris. The cell walls were collected at $20,000 \times g$. Electron micrographs showed the cell wall preparation to be free of whole cells. During the isolation procedure, most of the membrane fragments

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TABLE 1. Surface composition determined by XPS on whole cells and isolated cell walls of five gram-positive bacteria^a

Sample	C-(C,H)/C	C-(O,N)/C	C=O/C	COOH/C	O-C/C	O=C/C	O/C	N/C	P/C	K/C	S/C
Whole cells											
<i>Corynebacterium</i> sp. strain DSM 44016	69, 69	20, 20	11, 11	BDL, BDL	12, 12	12, 12	24, 24	9, 7	BDL, BDL	BDL, BDL	0.2, BDL
<i>Corynebacterium</i> sp. strain DSM 6688	71, 71	18, 19	10, 11	BDL, BDL	12, 12	12, 11	24, 23	8, 8	0.2, 0.3	0.3, 0.3	0.2, 0.2
<i>Rhodococcus erythropolis</i> A177	67	21	12	BDL	15	10	25	8	BDL	0.4	BDL
<i>Rhodococcus opacus</i> C125	73, 73	17, 17	10, 10	BDL, BDL	11, 11	9, 11	20, 22	8, 9	BDL, BDL	BDL, 0.2	BDL, BDL
<i>Bacillus brevis</i> ATCC 9999	49, 51	29, 28	22, 21	BDL, BDL	8, 9	25, 25	34, 33	20, 19	0.3, 0.3	0.2, 0.3	BDL, BDL
Isolated cell walls											
<i>Corynebacterium</i> sp. strain DSM 44016	70, 68	22, 23	8, 10	BDL, BDL	19, 17	7, 10	26, 26	6, 6	0.4, 0.4	0.4, 0.4	BDL, BDL
<i>Corynebacterium</i> sp. strain DSM 6688	69, 72	21, 19	10, 9	BDL, BDL	21	7	28, 30	6, 6	0.7, 0.7	BDL, BDL	BDL, BDL
<i>Rhodococcus erythropolis</i> A177	63, 64	26, 25	11, 11	BDL, BDL	25, 19	6, 10	31, 29	6, 7	BDL, BDL	BDL, BDL	BDL, BDL
<i>Rhodococcus opacus</i> C125	74, 73	19, 19	7, 8	2, BDL	31	BDL	31, 33	4, 4	BDL, BDL	BDL, BDL	BDL, BDL
<i>Bacillus brevis</i> ATCC 9999	58, 59	24, 25	14, 15	4, 4	12, 14	16, 18	28, 32	13, 15	0.4, 0.4	0.5, 0.6	0.4, 0.4

^a Data are expressed as atomic concentration ratios with respect to total carbon, multiplied by 100. When available, two sets of determinations are presented. BDL, below the detection limit (P/C, K/C, or S/C = 0.002).

were removed, as shown by a phospholipid analysis of the samples. The wet cell walls were stored at -20°C .

Sample preparation for XPS analysis. Prior to XPS analysis, the frozen cell wall samples were melted at room temperature. Cells and cell walls were harvested by centrifugation at $13,400 \times g$ for 10 min. They were washed three times by centrifugation in demineralized water. The pellets were resuspended in 2 ml of demineralized water and deposited in glass flasks (the diameter and height were both 2.5 cm) precooled in liquid nitrogen. The flasks were kept in liquid nitrogen for 15 min and stored at -80°C until freeze-drying. Freeze-drying was performed with an apparatus specially designed by Leybold. The temperature of the freeze-dryer shelf was set at -50°C during 3 h and then raised progressively to -5°C in about 15 h, maintained at -5°C during 6 to 12 h, and finally raised to 25°C . The flasks were stoppered and stored at room temperature in a desiccator containing silica gel. The dehydrated cell powder was homogenized with a spatula, introduced into a stainless steel trough with an inner diameter of 4 mm, gently compacted with a spatula, and pressed with a polyacetal cylinder (Delrin) cleaned with isopropanol, in order to obtain a smooth surface.

XPS analysis. The XPS analyses were performed with an SSI X-Probe (SSX-100/206) photoelectron spectrometer from Fisons, interfaced with a Hewlett-Packard 9000/310 computer. The pressure during analysis was between 2.5×10^{-6} Pa and 2.5×10^{-7} Pa. The spectrometer used monochromatized Al K_{α} X-ray radiation (1486.6 eV). The irradiated zone was an elliptic spot, with a shorter axis of 1,000 μm . The constant pass energies in the hemispherical analyzer were 150 and 50 eV for survey analysis and individual peak analysis, respectively. The flood gun energy was set to 6 eV, with a nickel grid placed 3 mm above the surface.

The spectra were recorded following the sequence C_{1s} , O_{1s} , N_{1s} , a survey spectrum, P_{2p} , S_{2p} , K_{2p} , Na_{1s} , Ca_{2p} , Mg (Auger $\text{KL}_{23}\text{L}_{23}$ peak) and, finally, C_{1s} again to check whether sample degradation took place. To assess the level of surface contamination, a sample of sorbitol was freeze-dried and submitted to analysis (C_{1s} and O_{1s} peaks and a survey spectrum) together with the analyzed samples.

The binding energies were determined by reference to the C_{1s} component due to carbon bound only to carbon and hydrogen, set at 284.8 eV. The background was subtracted linearly. Data treatment was performed with the ESCA 8.3 D software provided by the spectrometer manufacturer. The ratios of atomic concentrations were calculated by using the peak areas normalized on the basis of acquisition parameters and sensitivity factors proposed by the manufacturer (mean free path varying according to the 0.7th power of the photoelectron kinetic energy; Scofield cross sections [20]; transmission function assumed to be

constant). Elemental composition was expressed as atomic concentration ratios (mole/mole) relative to carbon. The C_{1s} and O_{1s} peaks were decomposed by using the software least-squares fitting routine with a Gaussian-Lorentzian ratio of 85/15. In a first fitting, the initial binding energies, the full width at half-maximum, and the intensity of the components were obtained without imposing constraints. A second fitting was performed by setting the full width at half-maximum of the components equal to the mean value of those obtained with the first fitting.

RESULTS

The carbon peaks were generally decomposed into three components, attributed to carbon bound only to carbon and hydrogen, C-(C,H), at a binding energy of 284.8 eV; to carbon singly bound to oxygen or nitrogen, C-(O,N), including ether, alcohol, amine, and amide, at a binding energy of 286.3 eV; and to carbon making one double bond or two single bonds with oxygen, C=O, including amide, carbonyl, carboxylate, ester, acetal, and hemiacetal, at a binding energy of 288.0 eV. In some samples, a weak component attributable to carboxylic functions was found at 289.1 eV. The broad oxygen peak was tentatively decomposed into two components. The first one, noted O-C, was attributed to hydroxide (C-OH), acetal, and hemiacetal (C-O-C), at a binding energy around 532.7 eV, and the second one was attributed to oxygen making a double bond with carbon (O=C), in carboxylic acid, carboxylate, ester, carbonyl, or amide, at a binding energy around 531.4 eV. Nitrogen appeared at a binding energy of 399.9 eV, attributable to unprotonated amine or amide functions (9, 17). Ca, Mg, and Na were always below the detection limit (atomic concentration ratio with respect to total carbon = 0.002); P, S, and K peaks were occasionally observed. Phosphorus was found at a binding energy of 133.5 eV and attributed to phosphate. The S peak (163.5 eV) was indicative of organic sulfide.

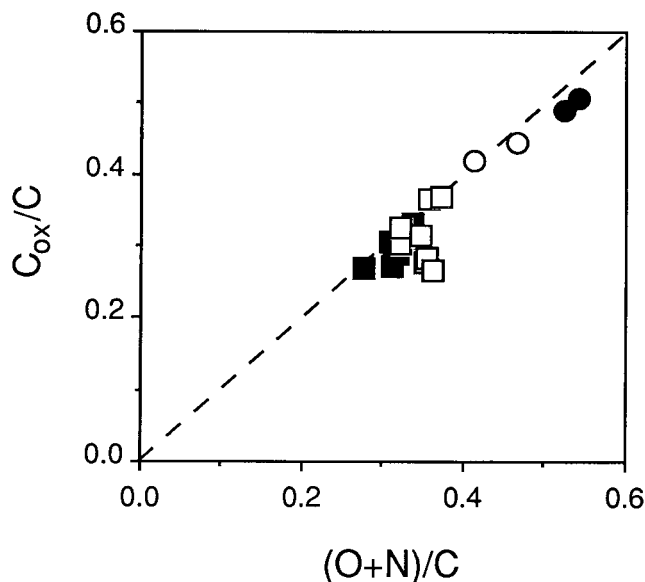


FIG. 1. Plot of the concentration (atomic concentration ratio with respect to total carbon) of carbon bound to oxygen or nitrogen (C_{ox}/C), deduced from the shape of the C_{1s} peak, as a function of the sum, $O+N$, deduced from the O_{1s} , N_{1s} , and C_{1s} peak intensities. Squares, coryneforms; circles, *B. brevis*; closed symbols, whole cells; open symbols, isolated cell walls. The interrupted line has a unit slope and zero intercept.

Table 1 presents the surface composition, in terms of atomic concentration ratios with respect to total carbon, determined by XPS on whole cells and on isolated cell walls. A duplicate set of XPS determinations, using the same culture but distinct freeze-drying, was carried out; the results show a good reproducibility of freeze-drying and XPS analysis. Both whole cells and isolated cell walls of *B. brevis* differed considerably from those of the coryneforms: they presented higher N/C , $(O=C)/C$, and $(C=O)/C$ ratios and a lower $(O-C)/C$ ratio. Systematic variations were observed between the results obtained with whole cells and those obtained with the corresponding isolated cell walls: the N/C ratio was higher for whole cells compared to isolated cell walls, and while the O/C ratio was generally similar, the shape of the O_{1s} peak was different, indicating a higher $(O=C)/C$ ratio and a lower $(O-C)/C$ ratio for whole cells compared to isolated cell walls.

Figure 1 presents a plot of the surface concentration, expressed as the atomic concentration ratio with respect to total carbon, of carbon bound to oxygen or nitrogen (C_{ox}/C), as a function of the sum of oxygen and nitrogen ($O+N$). The straight line of unit slope passing through the origin, which fits reasonably the experimental data, is in accordance with functional groups such as alcohol, hemiacetal, primary amine, non-primary amide, and ester. The slight excess of the sum $(O+N)/C$ compared to C_{ox}/C may reflect the presence of functions such as carboxylate and phosphate, in which carbon is not bound to oxygen or nitrogen in a 1:1 ratio.

Figure 2 shows that a good agreement is obtained between the concentrations of oxygen making a double bond with carbon ($O=C$), of carbon making one double bond or two single bonds with oxygen ($C=O$), and of nitrogen. The data are not far from a 1:1:1 proportion, which is typical of the amide function $O=C-N$. This may be attributed essentially to the presence of peptides in proteins and peptidoglycans and, to a lesser extent, to *N*-acetyl muramic acid in peptidoglycans (19). Figure 2 shows that the N/C , $(O=C)/C$, and $(C=O)/C$ atomic concentration ratios are about twice as high for both cells and cell walls of *B. brevis* (circles), compared to those of the coryneforms (squares). Moreover, these ratios are systematically higher for whole cells (closed symbols) than for isolated cell walls (open symbols).

DISCUSSION

The major constituents of the cell walls of gram-positive bacteria are peptidoglycan, teichuronic acids, (lipo)teichoic acids, (lipo)proteins, (lipo)polysaccharides, and sometimes lipids. The surface composition can therefore be modelled in terms of three classes of basic constituents (6): polysaccharides, peptides, and hydrocarbonlike compounds. Hydrocarbonlike compounds refer here to the main features of lipidic compounds. Compounds such as peptidoglycans are considered a combination of basic constituents, namely, peptides plus polysaccharides. The chemical composition of model constituents is presented in Table 2. This corresponds to $C_6H_{10}O_5$ for polysaccharides and to CH_2 for hydrocarbonlike compounds. The amino acid composition considered for peptides is that of the major outer membrane protein of *Pseudomonas fluorescens* OE 28.3 (4).

The molecular composition was computed with the following elemental concentration ratios:

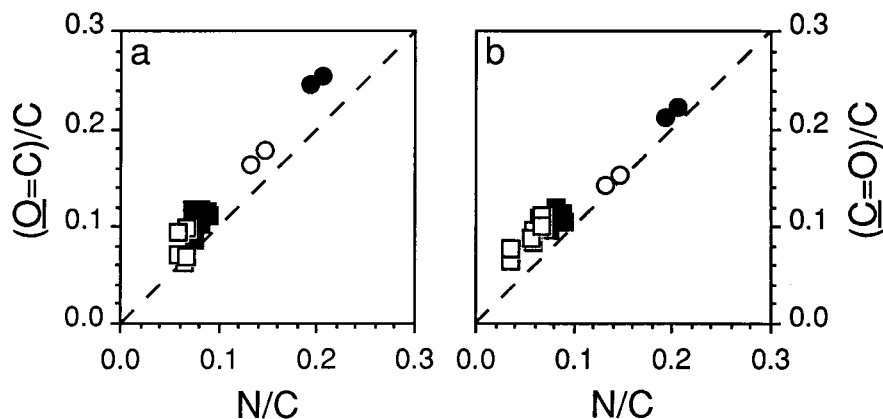


FIG. 2. Plot of the concentrations (atomic concentration ratio with respect to total carbon) of oxygen doubly bound to carbon ($O=C$) (a) and carbon making one double bond or two single bonds with oxygen ($C=O$) (b), as a function of nitrogen. Squares, coryneforms; circles, *B. brevis*; closed symbols, whole cells; open symbols, isolated cell walls. The interrupted line has a unit slope and zero intercept.

TABLE 2. Chemical composition of the model constituents considered for deduction of the surface molecular composition

Constituent	Atomic concn ratio					Carbon concn (mmol/g)
	C-(C,H)/C	C-(O,N)/C	C=O/C	O/C	N/C	
Polysaccharide	0.000	0.833	0.167	0.833	0.000	37.0
Peptide ^a	0.428	0.293	0.279	0.325	0.279	43.5
Hydrocarbon	1.000	0.000	0.000	0.000	0.000	71.4

^a Data were computed from the amino acid analysis of the major outer membrane protein of *P. fluorescens* OE 28.3 (4).

$$(N/C)_{\text{obs}} = 0.279 (C_{\text{PE}}/C)$$

$$(O/C)_{\text{obs}} = 0.325 (C_{\text{PE}}/C) + 0.833 (C_{\text{PS}}/C)$$

$$(C/C)_{\text{obs}} = (C_{\text{PE}}/C) + (C_{\text{PS}}/C) + (C_{\text{HC}}/C) = 1$$

where PS is polysaccharides, PE is peptides, and HC is hydrocarbonlike compounds.

Solving this system of equations provided the proportion of carbon associated with each molecular constituent: (C_{PS}/C) , (C_{PE}/C) , and (C_{HC}/C) . These proportions could then be converted into weight fractions, using the carbon concentration of each constituent (Table 2).

Figure 3 presents the modelled molecular composition of the surface of whole cells and of isolated cell walls calculated on the basis of elemental concentration ratios given in Table 1. Computations based on the three components of the carbon peak give similar results (data not presented). Both whole cells

and isolated cell walls of *B. brevis* present a surface composition markedly different from that of the other strains, the peptide content being about twice as high; this may be attributed to the presence of an S-layer on the surface of *B. brevis* cells (11, 22). A high concentration of hydrocarbonlike compounds (of the order of 40%) is observed for both whole cells and isolated cell walls of coryneforms, in accordance with the presence of mycolic acids (long-chain 2-alkyl-3-hydroxy-acids) in their cell walls (2). For *B. brevis*, part of hydrocarbonlike compounds might originate from residual membrane phospholipids (22).

Figure 4 presents the relationship between the surface concentration of model constituents and the water contact angle determined previously (23), for whole cells and isolated cell walls. Clearly, two groups of data can be distinguished. *B. brevis*, characterized by a high peptide concentration and low polysaccharide and hydrocarbonlike-compound concentrations, shows a contact angle in the range of 40 to 50°. Coryneforms, characterized by a higher hydrocarbonlike-compound concentration and a lower peptide concentration, show a contact angle above 60°. This is in agreement with previous observations (13) where the water contact angle of different bacterial species was directly correlated with the concentration of hydrocarbonlike compounds.

The peptidoglycan and protein contents of the cell walls of the five bacterial strains were recently determined (22) and are given in Table 3. Since peptidoglycan consists approximately of 50% glycan and 50% peptide, it is possible to assess the total peptide content and the glycan content originating from peptidoglycan (Table 3). Figure 5 shows the correlation between

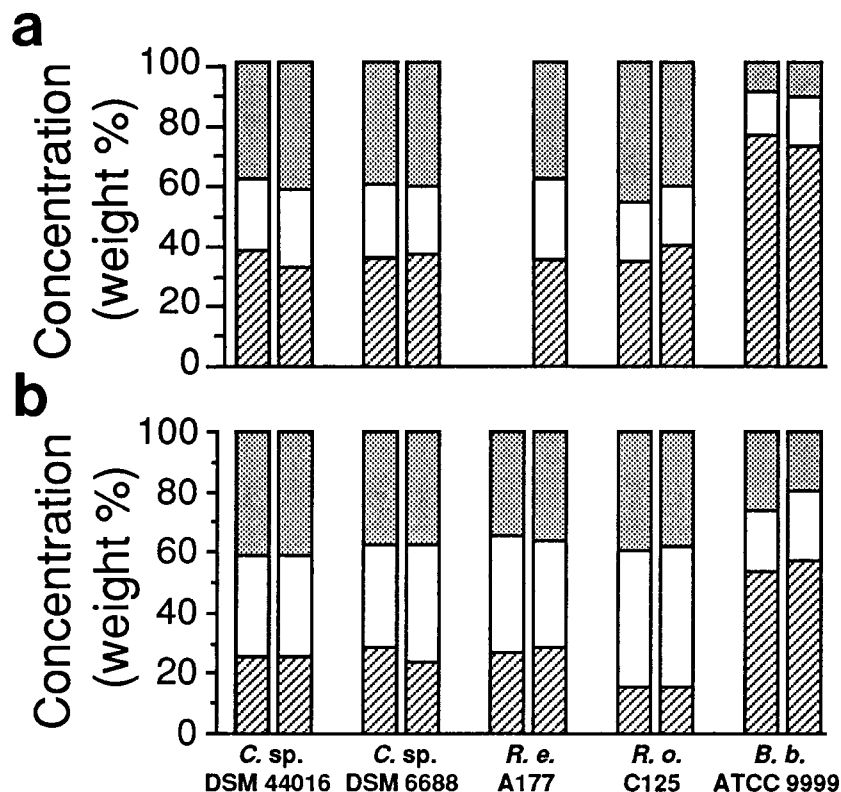


FIG. 3. Modelled molecular composition (weight percent) of the surface of whole cells (a) and isolated cell walls (b) deduced from XPS data. The computation is based on elemental O/C and N/C ratios. The strains used are as follows (from left to right): *Corynebacterium* sp. strain DSM 44016, *Corynebacterium* sp. strain DSM 6688, *R. erythropolis* A177, *R. opacus* C125, and *B. brevis* ATCC 9999. ▨, HC compounds; □, polysaccharides; ▤, peptides.

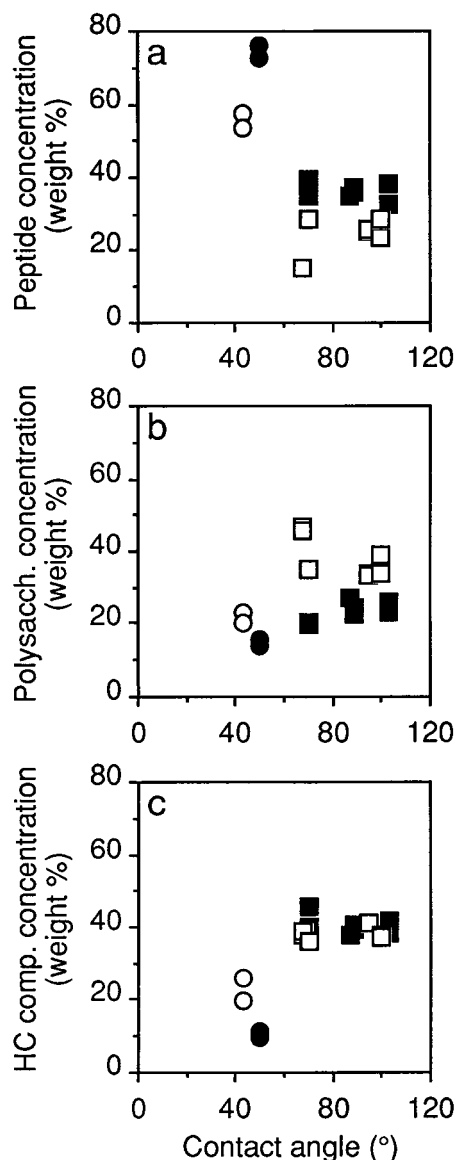


FIG. 4. Relationship between the molecular composition deduced from XPS data—peptide (a), polysaccharide (b), and hydrocarbonlike compound (c)—and the water contact angle. Closed symbols, whole cells; open symbols, isolated cell walls; squares, coryneforms; circles, *B. brevis*.

the peptide concentration deduced from XPS data and the total peptide content deduced from biochemical analysis. The agreement between the two parameters supports the validity of modelling XPS data to approach the molecular composition.

From Fig. 3b and Table 3, it follows that the content of polysaccharides which are not in the form of peptidoglycan is about 20%. The occurrence of polysaccharides, such as arabinogalactan, is indeed expected in the cell wall of coryneform bacteria (2). For *B. brevis*, glycans are also expected in association with proteins (glycoproteins) in the S-layer (22).

For all strains, significant differences are observed in the peptide/polysaccharide ratio determined by XPS when comparing whole cells with isolated cell walls: a lower peptide content and a higher polysaccharide content are observed systematically for the cell walls. The *B. brevis* cell wall is multi-layered: the outermost (electron-dense) layer is a protein rich

TABLE 3. Concentrations of the peptidoglycan and protein in the isolated cell walls (22) and deduced values for total peptide and for glycan from peptidoglycan

Strain	Indicated value (concn [dry weight %]) of cell wall constituent			
	Experimental		Deduced	
	Peptidoglycan	Protein	Total peptide	Glycan
<i>Corynebacterium</i> sp. strain DSM 44016	23	14	25.5	11.5
<i>Corynebacterium</i> sp. strain DSM 6688	27	14	27.5	13.5
<i>Rhodococcus erythropolis</i> A177	24	10	22	12
<i>Rhodococcus opacus</i> C125	31	7	22.5	15.5
<i>Bacillus brevis</i> ATCC 9999	5	56	58.5	2.5

S-layer, whereas the inner layer is mainly made of peptidoglycan. The higher polysaccharide content obtained for the isolated cell walls may thus be explained by the fact that the exposed surface, which is analyzed by XPS, is partly outer- and partly inner-layer material. The XPS analysis of whole cells is thus more relevant to investigate cell surface composition, compared to analysis of cell walls by XPS and by biochemical methods as well.

XPS analysis of whole cells and biochemical analysis of isolated cell walls should also be compared in terms of possible artifacts occurring from sample preparation. To achieve high vacuum conditions required for XPS analysis, microbial cells are freeze-dried prior to introduction into the spectrometer; cell disruption or migration of intracellular components to the cell surface may be excluded, but reorganization of cell surface polymers may take place (7). On the other hand, losses of constituents and contamination by intracellular compounds may occur during cell wall isolation. In particular, destruction of S-layers may result from using a high concentration of chao-

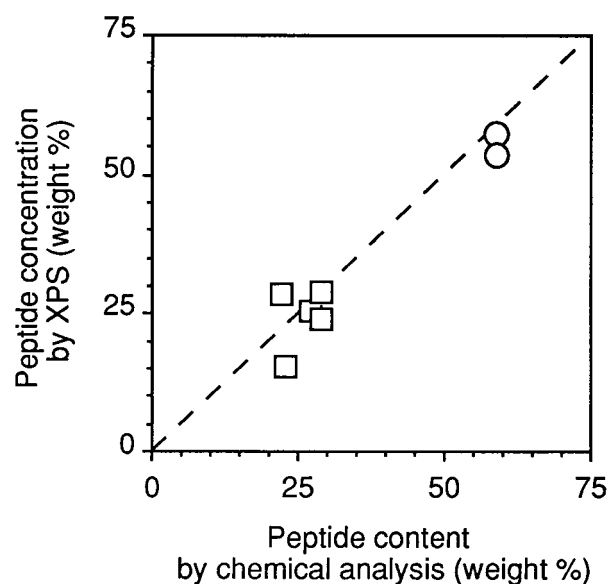


FIG. 5. Relationship between the peptide concentration determined by modelling XPS data and the total peptide content obtained by biochemical analysis, for isolated cell walls of coryneforms (squares) and *B. brevis* (circles).

tropic agents such as urea or guanidinium hydrochloride (11). Removal of part of the S-layer of *B. brevis* may be responsible for a lower peptide/polysaccharide ratio, as observed by XPS. This hypothesis could be investigated further: isolated S-layer subunits from numerous bacterial species have indeed the ability to reassemble into two-dimensional arrays in suspension or at various interfaces upon removal of the disrupting agents used for their isolation (11, 14, 15).

XPS analysis of microbial cells requires heavy equipment, but the time-consuming work of cell wall isolation for biochemical analysis can be avoided. The relative precision of XPS analysis is currently better than 10% for major elements and functions and is mainly determined by the variability of the biological material (17). Minor elements (P, K, S, etc.) can be detected down to a molar concentration of about 0.002 relative to carbon. In contrast with biochemical analysis, XPS is not able to detect individual compounds; however, its application to whole cells provides information directly on the outermost cell surface and not on the entire cell wall, which makes it more relevant to the understanding of the surface properties of microbial cells and their behavior at interfaces (adhesion, aggregation, and flotation).

Conclusion. Comparison of XPS analysis and biochemical analysis of isolated cell walls of five gram-positive bacteria supports the validity of using XPS to approach the overall macromolecular composition of the bacterial cell surface. This is performed by converting the XPS elemental data in terms of the main classes of biochemical constituents. XPS analysis of whole cells or isolated cell walls and biochemical analysis of isolated cell walls are consistent in showing differences of composition between *B. brevis* and coryneforms. *B. brevis* has a much higher peptide concentration due to the presence of an S-layer. However, systematic differences between XPS analysis of whole cells and that of isolated cell walls point to advantages of XPS analysis over biochemical analysis of isolated cell walls: the outermost cell surface (2- to 5-nm-thick layer) is directly characterized, and the alteration by chemical treatments is avoided. This makes XPS a valuable tool to investigate the surface composition of microorganisms in relation to their behavior at interfaces.

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