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Cell Wall Composition of *Bacillus subtilis* Changes as a Function of pH and Zn^{2+} Exposure: Insights from Cryo-XPS Measurements

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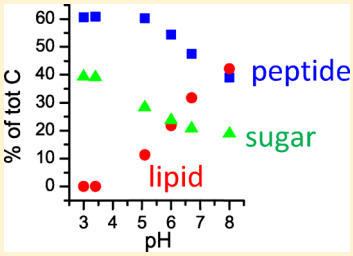
Supporting Information

ABSTRACT: Bacteria play an important role in the biogeochemical cycling of metals in the environment. Consequently, there is an interest to understand how the bacterial surfaces interact with metals in solution and how this affects the bacterial surface. In this work we have used a surface-sensitive analysis technique, cryogenic X-ray photoelectron spectroscopy (cryo-XPS), to monitor the surface of *Bacillus subtilis* cells as a function of pH and Zn^{2+} content in saline solution. The objective of the study was twofold: (1) to investigate the agreement between two data treatment methods for XPS, as well as investigate to what extent sample pretreatment may influence XPS data of bacterial samples, and (2) to characterize how the surface chemistry of bacterial cells is influenced by different external conditions. (1) It was found that the two data treatment methods gave rise to comparable results. However, identical samples analyzed fast-frozen or dry exhibited larger differences in surface chemistry, indicating that sample pretreatment can to large extents influence the obtained surface composition of bacterial samples. (2) The bacterial cell wall (in fast-frozen samples) undergoes dramatic compositional changes with pH and with Zn^{2+} exposure. The compositional changes are interpreted as an adaptive metal resistance response changing the biochemical composition of the bacterial cell wall. These results have implications for how adsorption processes at the surface of bacterial cells are analyzed, understood, modeled, and predicted.



INTRODUCTION

Metal sequestration of bacteria plays an important role in the biogeochemical cycling of metals in the environment. In order to be able to understand and predict such processes, there have been extensive attempts to model and understand the interactions between protons, metal ions, and bacterial surfaces as well as to characterize them using spectroscopy.[1−9] Previous studies of metal adsorption onto bacterial surfaces have suggested that the same types of functional groups are involved in both Gram-positive and Gram-negative bacteria in metal sequestration.[4,10] For Cd^{2+} it was found that phosphoryl and carboxyl binding play a large role in higher or intermediate loading conditions for Gram-positive bacteria, but at lower loadings the sulfuryl and carboxyl groups become important (3 ppm) and at low loadings the sulfuryl are the main binding sites (at 1 ppm).[4] The same functional groups have been identified in Zn^{2+} binding to Gram-negative bacteria.[11] Adsorption of Cd^{2+} and Pb^{2+} has been reported onto carboxylic and phosphonate groups in peptidoglycan and teichoic acids of the cell wall of Gram-positive bacteria,[12,13] and it has been suggested that extracellular substances play a large role in metal sequestration.[11,14] There also have been suggestions of a universal adsorption edge for metals onto all types of bacteria.[15−17] However, there are implications that the current models are too simplistic and that, in fact, the cell wall changes e.g. at lower pH values to allow for a larger number of binding sites for cations.[18] Consequently, in order to address this issue, it is important to understand the dynamics of the bacterial cell wall as a function of external parameters such as pH and metal ion exposure.

X-ray photoelectron spectroscopy (XPS) is a surface-sensitive analysis method that has been used to analyze the chemical composition of bacterial cells.[19−22] The depth-of-analysis of this method allows for studies of only the outermost part of the bacterium. If the cells are analyzed fast-frozen (cryo-XPS), water will remain in the structure, which is believed to preserve some of the architecture of the cell wall.[20,21] The cell walls in Gram-positive bacteria and Gram-negative bacteria have different compositions. The Gram-negative cell wall consists of a plasma membrane, a periplasmic space with a thin layer of peptidoglycan, and an outer membrane consisting of phospholipids on the inside and lipopolysaccharides (LPS) on the outside. Proteins are present in all these layers of the cell wall. Cryo-XPS analysis of intact Gram-negative bacteria is assumed to provide information from the outer membrane and the thin peptidoglycan layer in the periplasmic space.[21] Gram-positive bacteria have a cell wall consisting of a plasma membrane and, outside of that, a thick peptidoglycan layer (30−100 nm) containing teichoic acids, lipoteichoic acids, and proteins.[23] The thickness of the peptidoglycan layer in Gram-positive bacteria suggests that XPS here only probes the peptidoglycan layer and its constituents. For both Gram-negative and Gram-positive bacteria, surface appendages and/

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or extracellular substances, such as flagella, pili, and capsules, will influence the XPS spectra to some extent depending on their quantity. Consequently, bacteria with flagella and pili (or fimbriae) may display higher peptide content, and the presence of a capsule is expected to increase the polysaccharide content of the spectra.

In this work we have used cryo-XPS to investigate how the bacterial cell wall of Gram-positive *Bacillus subtilis* changes with pH and with exposure to Zn(II). *B. subtilis* is a common soil bacterium that has been reported to tolerate high concentrations of heavy metals such as Zn(II)[24] and is a suitable model organism, since it is expected to play a large role in metal biogeochemical cycling in soil environments. We show that the dramatic changes occurring at the surface of bacterial cells can be followed using cryo-XPS and that this technique can be used as a tool to better understand how bacterial surfaces and metal ions interact in the environment. We have compared two data analysis methods for "unmixing" XPS data to extract the composition of the bacterial cell wall with respect to lipids, sugars, and peptides. This comparison aims to establish how well the two data treatment methods agree for dehydrated samples, to allow better comparisons between literature studies using XPS analyses. Furthermore, we have investigated some consequences of sample pretreatment in order to study possible effects of the dehydration process of bacterial samples and in an attempt to evaluate sample pretreatment methods.

EXPERIMENTAL SECTION

Reagents. All solutions were prepared by using deionized and boiled water. NaCl (Merck) was dried overnight at 180 °C and used to adjust the ionic strength to 0.1 M Na(Cl) in all experiments. Stock solutions of HCl (Fisher p.a.) were standardized against Trizma base tris(hydroxymethyl)aminomethane. NaOH (Merck p.a.) stock solutions were standardized against a primary standard HCl solution. $ZnCl_2$ (Merck) and $Zn(NO_3)_2{\cdot}6H_2O$ (BDH Chemicals) were used without further purification to prepare respectively the solutions used for the adsorption experiments and for the atomic absorption spectroscopy (AAS) calibration curve. Both were standardized with EDTA using xylenol orange as indicator.[25]

Bacterial Growth. A *Bacillus subtilis* strain (ATCC 6633) was grown at 30 °C in Luria−Bertani broth (composition for 1 L of culture medium: yeast extract = 5 g, tryptone = 10 g, NaCl = 5 g) under aerobic conditions on an orbital shaker. The cells were collected by centrifugation at 2880*g* for 20 min at the end of the exponential growth phase, washed twice with physiological solution (NaCl 0.9%), and resuspended in 0.1 M NaCl ionic medium. The optical density (OD600) of this parent bacterial suspension was measured (spectrophotometer UV-1201 V, Shimadzu), and this value was correlated to the dry weight of bacterial cells by using a one-point calibration procedure.

Preparation of Bacterial Samples at Different pH Values. Wet paste samples at different pH values were generated by adding HCl and NaOH solutions to resuspended bacterial solutions, and aliquots were removed at desired pH values. Measurement of pH was done using a pH electrode that was calibrated with a one-point calibration procedure in an acidic solution of known concentration in 0.1 M NaCl at 25 °C. The bacterial cells were separated from the supernatant solutions by centrifugation at 10 000 rpm for 6 min.

Zn(II) Adsorption onto *Bacillus subtilis*. Zn(II) adsorption by *Bacillus subtilis* suspensions was studied in batch experiments performed at 25 °C ± 1 and in 0.1 M Na(Cl) ionic medium. Samples from the bacterial parent suspensions were transferred into 10 mL tubes, and the pH was varied by adding aliquots of HCl and NaOH solutions prepared in the same ionic medium from automatic burets. Aliquots of the $ZnCl_2$ solution in 0.1 M Na(Cl) were added at the end in order to have a total Zn(II) concentration in the samples corresponding to a ratio $[Zn^{2+}]$/bacterial biomass of 0.12−0.14 mmol/g (dry weight) (corresponding to a total concentration Zn(II) in solution approximately 1 mM). The samples were placed on an end-over-end rotator and centrifuged for 20 min at 2880*g* after 24 h equilibration time at 25 °C ± 1 and remeasuring of pH. Following centrifugation, the supernatants used for AAS analysis were separated from the paste, filtered through 0.22 μm membranes (MILLEX GS filters, MILLIPORE), acidified, and stored in the refrigerator (4 °C) until analysis.

XPS Analysis. Two series of batch samples were analyzed by XPS in parallel as a function of pH, and they were prepared as follows: from each parent suspension of bacterial cells two samples were transferred into 10 mL tubes. Equal aliquots of acid or base were added to the two samples in parallel, and an aliquot of the $ZnCl_2$ solution was added to one. The final volume of the two samples was adjusted to the same value by using the 0.1 M NaCl solution. While the concentration of the bacterial cells was the same in each of the two replicate samples, this value varied from 5.7 to 9.2 g/L dry weight in all the batch samples prepared at different pH values, and therefore the aliquots of the $ZnCl_2$ solution were adjusted in order to obtain a ratio between $[Zn(II)]_{tot}$/BS(g/L dry weight) in the range of 0.13−0.17. The samples were equilibrated for 24 h at 25 °C on an end-over-end rotator and centrifuged at 2880*g* for 20 min after remeasuring pH. The equilibrium time was chosen based on previous potentiometric studies of this system performed in our lab, which showed that equilibrium was reached before 24 h.[26] The supernatant solutions were discarded, and the bacterial pellets were analyzed as fast-frozen wet pastes using a precooling procedure described elsewhere.[20] The XPS spectra were collected with a Kratos Axis Ultra DLD electron spectrometer using monochromated Al Kα source operated at 150 W. An analyzer pass energy of 160 eV was used for acquiring wide spectra and a pass energy of 20 eV for individual photoelectron lines. The surface potential was stabilized by the spectrometer charge neutralization system. The binding energy (BE) scale was referenced to the C 1s peak aliphatic carbon at 285.0 eV. Thereafter, the compositions of C 1s spectra were modeled using the spectral components from the multivariate analysis of C spectra described previously.[21] To compare spectra acquired from frozen and dry pastes, some samples were left to slowly warm to room temperature and sublimate the water inside the spectrometer and were subsequently analyzed once more in the same position the following day.[27] This second measurement will be denoted as warmed or dry samples in the text.

XPS Data Treatment. In C 1s spectra from Gram-positive bacterial samples, aliphatic carbon can be distinguished as a peak at 285.0 eV. Another peak at 286.5 eV corresponds to C bound to O or N through a single bond. A peak at 288.2 eV corresponds to carbon in a peptide bond, bound to oxygen through a double bond (carbonyl group), or a C with two O neighbors, and a small peak at 289.3 eV corresponds to carboxylic acid. These different carbon atoms exist in several different organic substances in the cell wall, and in order to derive the amount of each substance, one needs to separate the contributions from each substance in the spectral mixture, a process often described as "unmixing". In multivariate analysis of C spectra this is done by assuming that the measured spectra are composed of a sum of each substance spectrum at different ratios (or percentages of the total C peak). Mathematical "unmixing" of the C spectra has previously been performed on a large set of Gram-negative bacterial samples including standards for wall components.[21] The result was that all spectra could be described as linear combinations (mixtures) of three mathematical (but chemically realistic) components that resemble the spectra of lipid, polysaccharide, and peptide. The component denoted as peptide included both protein and peptidoglycan since the XPS spectra of these two substances are too similar to "unmix" mathematically.[21] The obtained multivariate model can be used to predict the composition of other types of biological samples as long as the sample composition consist of similar types of organic compounds. In this work we have used this model successfully on Gram-positive bacteria to better understand how the dynamic bacterial surface changes with pH and in the presence of Zn(II). For Gram-negative bacteria the components in the multivariate model developed[21] predicted lipids in the outer membrane (lipid), protein,

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and peptidoglycan (peptide) as well as lipopolysaccharides and polysaccharides on glycosylated proteins (polysaccharide). For Gram-positive bacteria the cell wall composition differs to some extent, and instead the lipid component in the multivariate model illustrates changes in the aliphatic part of lipoteichoic acid, the peptide fraction changes in proteins and peptidoglycan (similar as for Gram-negative bacteria), and the polysaccharide component would represent polysaccharides in teichoic and teichuronic acids, polysaccharide on glycosylated proteins, and also polysaccharide in lipoteichoic acid.

The multivariate model is not the first attempt described in the literature to extract the substance composition of the bacterial cell wall from XPS data. Previously, Rouxhet and co-workers[19,28,29] developed equation systems with ratios between different components in XPS spectra, obtained from curve-fitting, that allow for calculations of the ratios of protein (C_{Pr}/C), polysaccharide (C_{PS}/C), and hydrocarbon-like compounds (C_{HC}/C) to total carbon using the following equation system:

$$[N/C]_{obs} = 0.279(C_{Pr}/C) \quad (1)$$

$$[O/C]_{obs} = 0.325(C_{Pr}/C) + 0.833(C_{PS}/C) \quad (2)$$

$$[C/C]_{obs} = 1 = (C_{Pr}/C) + (C_{PS}/C) + (C_{CH}/C) \quad (3)$$

This scheme works well as long as there are no interfering substances for example in the nitrogen or oxygen spectra. Consequently, for frozen samples where the structural water remains, other methods were needed. The multivariate "unmixing" of components contributing to the C 1s spectra, used here, was developed in response to the need to estimate and predict the composition of the bacterial cell wall in cryo-XPS.[21] The unmixing performed using the multivariate components can be seen as a version of eq 3 previously described by Rouxhet et al.,[29] and consequently the outputs from the two methods should be comparable. However, the multivariate model is expected to be less subjective to differences in peak-fitting procedures between different experimentalists as the fitting in the multivariate model is automatically done using predefined mathematical components for each substance.[21]

Statistics. Statistical analysis (ANOVA) was performed using Origin 8.1 (Origin Lab Corporation).

RESULTS AND DISCUSSION

Analysis of XPS spectra: Comparison between Evaluation Models. The output from the spectral "unmixing" performed using the equation system and the multivariate analysis was compared by allowing a frozen sample sublimate the water in vacuum and remeasure the dry sample the following day. Thereafter, the spectra from dry samples were analyzed using both data treatment methods. The two methods gave similar results for the three components in *Bacillus subtilis* samples (Figure 1) showing that both methods could be used to monitor and describe changes in the surface composition of bacterial cells in dehydrated bacterial samples. The largest difference between the methods was observed for the lipid component where it seems the equation system gives higher lipid content than the multivariate model does in samples with low lipid contents, but lower lipid content in samples with a higher amount of lipids. The reason for the difference in lipid content could be that in the equation system the lipid fraction is determined as the remaining C signal after subtraction of protein and polysaccharide. This could mean that the entire error in the "fit" will end up in the lipid content when using the equation system, whereas in the multivariate analysis the error can be distributed between different components and is reflected in the overall fit of the model to the spectra.

However, despite the small variations from ideal linearity in the case of the lipid fraction, the obtained regression lines suggest a relatively good agreement between the methods, with

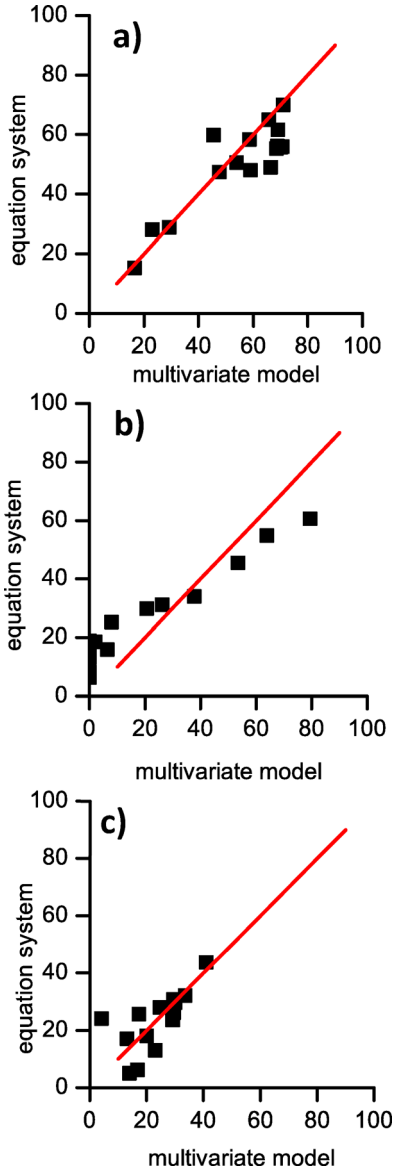

Figure 1. Plots showing the correlation between the percentages of each component calculated using equation systems and multivariate analysis of warmed samples (a) peptide, (b) lipid, and (c) polysaccharide. Please note that the red lines represent an identical percentage between the two data treatment methods and not a regression line.

slope (y) of 0.74 ± 0.23 (intercept m = 5.9 ± 5.7, correlation coefficient r^2 = 0.43, and probability p = 0.0068) for polysaccharide, y = 0.61 ± 0.04 (m = 14.1 ± 1.4, r^2 = 0.94, p = 6.3 × 10^{-9}) for lipid, and y = 0.72 ± 0.10 (m = 10.7 ± 5.9, r^2 = 0.77, p = 5.5 × 10^{-6}) for peptide. This agreement suggests that the data obtained using the two methods are comparable and can be used side-by-side on dehydrated samples. Both methods should be possible to use on any type of bacterial sample in order to extract biochemical information about the surface composition of bacterial cells and how it is influenced by environmental conditions. However, for frozen samples that have not been dehydrated, the method with the equation system cannot be used due to the presence of water in O 1s spectra.

Comparison between Sample Preparation Methods. Samples that were analyzed frozen as well as dry, i.e. after

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sublimation of water in the vacuum, showed differences in composition (Figures 2 and 3), especially for the peptide and

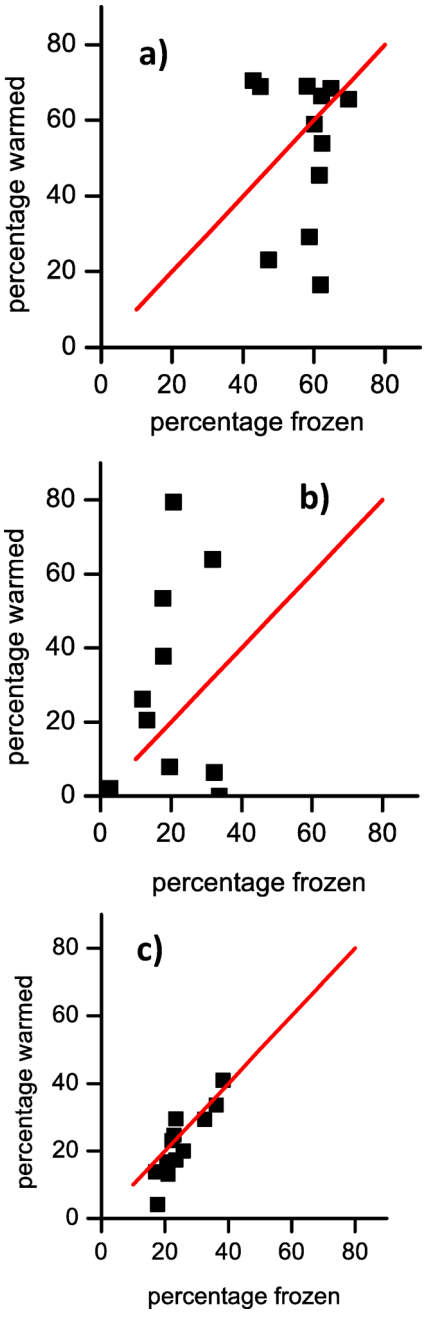

Figure 2. Differences in percentages between frozen and dry (ice sublimated) samples with respect to (a) peptide, (b) lipid, and (c) sugar content. The sugar content remained the same in dry samples whereas the lipid and peptide contents change. Linear regression of the data points in (a), (b), and (c) give rise to correlation coefficient r^2 of −0.1, −0.02, and 0.75, respectively. The red line represents an identical ratio between samples and not a model line.

lipid component (no correlation seen in Figure 2a,b). The data for polysaccharide could, however, still be described by a regression line with $y = 1.29 \pm 0.22$, $m = 10.1 \pm 5.8$, $r^2 = 0.75$, and $p = 1.8 \times 10^{-4}$, indicating that the content of polysaccharide was not altered to the same extent in the dehydration process. In many samples, the lipid content increased in the dried sample whereas the peptide component

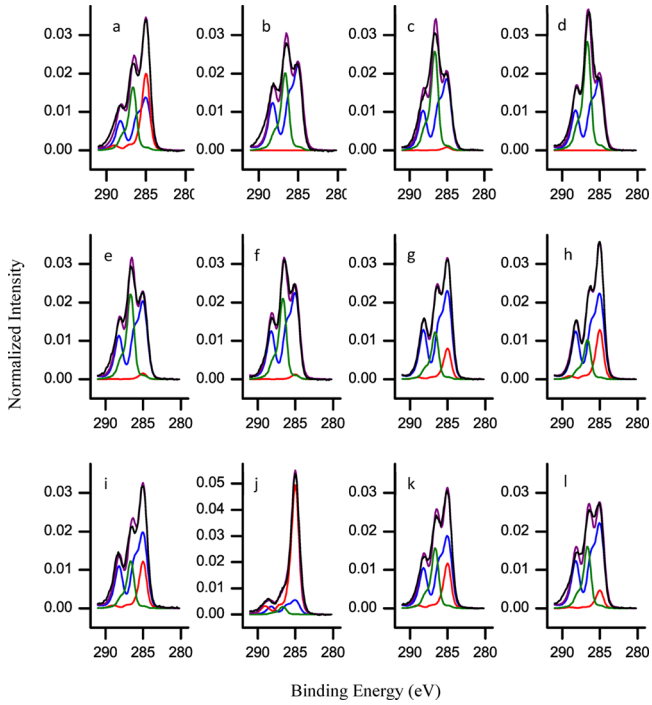

Figure 3. Fit between data and the multivariate model of the C 1s spectra: (a) pH 1.7 frozen, (b) pH 1.7 dry, (c) pH 3.4 frozen, (d) pH 3.4 dry, (e) pH 4.5 frozen, (f) pH 4.5 dry, (g) pH 6 frozen, (h) pH 6 dry, (i) pH 6 frozen, (j) pH 6 dry, (k) pH 8.2 frozen, and (l) pH 8.2 dry. The data are represented by a black line and the fit by a purple line. The components are shown in blue (peptide), red (lipid), and green (polysaccharide). All samples are *Bacillus subtilis*. Some differences can be seen between spectra of frozen samples and dried samples (sublimated). One sample from pH 6 displayed a dramatic change in lipid content (i, j).

decreased. The changes induced from drying are of course a result of the disappearance of water. This could give rise to migration of cell wall components during drying,[30] as have been seen in tissue samples, but could also be a result of structural reorganization of macromolecules where more hydrophilic sections could reorganize to become more buried as the water is removed from the surface. One example of such reorganization could be denaturation of surface proteins to expose more hydrophobic core regions while hiding hydrophilic side chains deeper into the surface. Possibly some dramatic changes could also be signs of rupture of the cell wall to expose the lipid-rich plasma membrane. However, it is important to stress that when dehydration is done carefully, the microstructure of bacterial cells has been reported to remain intact for *Bacillus subtilis* after drying[31] and not show signs of cell wall rupture. The disappearance of water could also make the bacterial cells shrink, allowing for analysis deeper into the bacterial surface. The exact nature of the reorganization and changes at the bacterial surface as a result of drying can only be speculated, but whatever process is causing the large change, these results indicate that drying bacterial cells may alter the surface composition significantly. This suggests that, unless great care is taken during the dehydration process, it is preferable to analyze bacterial cells in a frozen form. Analyzing samples as frozen bacterial pellets, furthermore, reduces sample preparation and allows for a shorter overall analysis procedure. Consequently, the remaining data presented here are from frozen samples to avoid any artifacts from the drying process.

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pH-Induced Changes in Bacterial Surface Composition. Large changes in cell wall composition (in fast-frozen samples) were observed throughout the investigated pH range in the untreated C 1s cryo-XPS spectra (Figure 3 and Supporting Information Figure 1) as well as in ratio of different substances predicted by the multivariate model (Figure 4).[21]

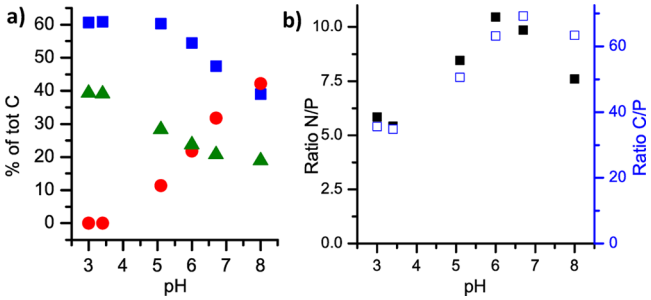

Figure 4. Compositional changes of the cell wall as a consequence of pH changes. (a) Predicted composition from XPS C 1s spectra, blue squares represents peptide, green triangles represents sugar (teichoic and teichuronic acids), and red circles represents lipid (lipoteichoic acids). (b) Changes in N/P (black filled squares) and C/P (empty blue squares) atomic ratios with pH (from XPS measurements).

At low pH and in dry samples the multivariate model was not able to predict a small shoulder appearing on the high binding energy side of the carbon spectra. This component at 289.5 eV would correspond to protonated carboxylic groups that have a slightly higher binding energy than the nonprotonated.[32] Carboxylic groups on bacteria have previously been shown to become protonated below pH 4,[26] which corresponds well to the appearance of the slight increase of this shoulder in C spectra at low pH.

The changes observed in the cell wall composition (Figure 4) represent a sum of several changes occurring in the bacterial cell wall with changes in pH. Bacteria such as *Bacillus subtilis* have been reported to alter their gene transcription and protein production depending on the pH they are exposed to. For example, membrane-bound protein complexes are up-regulated at high pH in *Bacillus subtilis* and several enzymes that reduce acidity and promote metal transport out of the cell are up-regulated at low pH.[33] Thus, the decrease in peptide (Figure 4a) with pH could suggest that these processes up-regulate protein synthesis to a higher level at low pH under the specific conditions used here (nutrient poor saline). Other changes that have been reported are changes in building blocks of the cell wall with pH. For example, the content of teichoic acid (phosphate rich) has been reported to increase at low pH in the membrane of Gram-positive bacteria whereas the teichuronic acid (lacking phosphate) is said to increase with increasing pH.[34] This would probably not produce dramatic changes in the C spectra but could influence the relative amount of phosphate. Indeed, changes in the obtained N/P and C/P ratios could be observed with pH (Figure 4b). However, since these changes occur simultaneously to changes in protein expression levels, they are difficult to unambiguously interpret. For example, a simultaneous increase in teichuronic acid and decrease in protein content could result in a constant N/P ratio.

Bacillus subtilis generally inhabits environments with pH between 6 and 9.[33] In samples at very low pH the surface composition is distinctly different from samples at higher pH which could be due to spore formation triggered by the acidity.[33] The spore coat is rich in protein with smaller amounts of carbohydrates and lipids,[35] which corresponds well to the composition obtained from XPS at low pH. Thus, the observed changes appear to be in line with previously reported bacterial responses to changes in pH and/or metal content in their surroundings by excreting substances as well as by altering their cell wall composition to cope with this external stress.[33,36,37]

Zn(II) Adsorption onto *Bacillus subtilis*. The presence and adsorption of Zn(II) to the bacterial cell were studied by following the concentration of Zn(II) in solution using atomic absorption spectroscopy (AAS) (Figure 5a) and at the surface using XPS (Figure 5b).

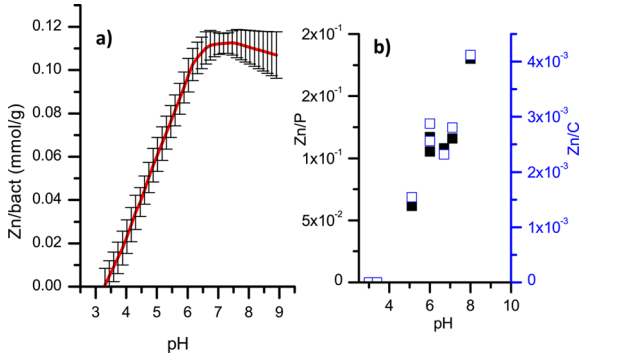

Figure 5. (a) Average adsorption of Zn(II) onto *Bacillus subtilis* as a function of pH (ratio between Zn(II)/dry weight biomass 0.12 mmol/g) at 24 h equilibration time. The line represents an average of four different experiments with varying amounts of bacteria. Error bars represent standard deviation. (b) Zn(II) accumulation at the surface of the sample as illustrated by the Zn/P (filled squares left *y*-axis) and Zn/C (empty squares right *y*-axis) atomic ratios. Points at low pH with a ratio of 0 represent samples where Zn could not be detected with XPS.

Figure 5 shows that as Zn(II) disappears from solution it is accumulated onto the bacterial surface, giving rise to an absorption edge between pH 4 and 7 similarly to what has previously been reported for *Bacillus subtilis*.[18,38] However, it was not possible to know if all Zn(II) disappearing form solution is adsorbed or precipitated at the surface of the bacterium or if some was accumulated inside the cells as a response to Zn(II) resistance mechanisms.[14] Equilibrium calculations of zinc hydroxide precipitation from solution indicated that at concentrations of 1 mM a precipitate would be expected above pH 7, suggesting that the loss of Zn from solution at lower pH values is a result of other processes than hydroxide precipitation, as has previously been reported.[18,38]

The cryo-XPS results, including the multivariate "unmixing", showed that the bacterial cell surface composition was affected by the presence of Zn(II) (Figures 5 and 6). The compositional trends with pH observed in absence of Zn(II) (Figure 4) were dampened, and the data appeared more scattered (Figure 6a), illustrating that the bacterial surface responded in some way to the presence of Zn(II). Interestingly, the data for samples at low pH were very similar to those without the presence of Zn(II), supporting the hypothesis of spore formation at low pH in both conditions.

The surface charge behavior, as reflected in Na/Cl ratio, also seemed to change following Zn(II) exposure (Figure 6b). In the absence of Zn(II), this ratio remained close to 1 throughout the pH range, reflecting the constant zeta potential (and

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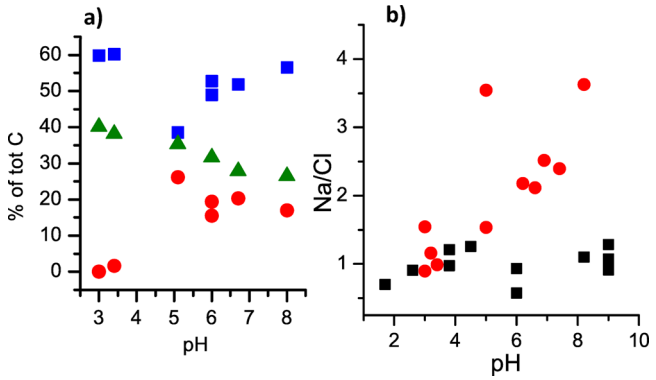

Figure 6. (a) Surface composition of *Bacillus subtilis* exposed to Zn(II). Blue squares represents peptide, red circles lipid, and green triangles sugar. (b) Change in Na/Cl ratio with pH for frozen bacterial samples (black squares) and frozen bacterial samples exposed to Zn(II) (red circles).

electrophoretic mobility) between pH 5 and 9 in the system without Zn.[26] Since the cells are negatively charged, we should have an excess of Na at the surface. Consequently, the 1:1 ratio observed using XPS could indicate that there are some processes actively governed by the live bacterial cells lowering the overall surface charge, possibly involving $Na^+ \leftrightarrow H^+$ exchange. However, in the presence of Zn(II) the Na/Cl ratio increases with increasing pH in solution, indicating an increase in negative charge at the surface of the bacteria following Zn(II) accumulation (Figure 6b) and loss of the hypothesized active proton exchange process. A possible explanation for the increased negative charge above pH 7 could be that Zn(II) accumulates at the surface in the form of a hydroxide precipitate that exposes a net negative surface charge balanced by Na^+ counterions. However, the chemical speciation of the accumulated Zn(II) at the surface was not possible to deduce from the XPS analyses due to the low Zn concentration and is a topic for further studies.

Metal ions such as Zn(II), Ni(II), Co(II), and Cu(II) are nutrients for the bacterium in low concentrations but are toxic at higher concentrations. Live bacteria exposed to metals will consequently start to up-regulate different metal resistance mechanisms such as exclusion of metals by barriers in e.g. the cell wall, metal sequestration intra- and extracellularly, efflux pumps transporting metals out of the cell, and metal detoxification by enzymes, reducing the sensitivity of targets in the cell to metals.[39] Many of these mechanisms relate to the cell wall of the bacterium and its composition and result in changes in porins or efflux pumps in the membrane or construction of protective layers on the outside of the cell wall. These processes have been suggested to be among the most important for allowing bacteria such as *B. subtilis* to survive in heavy-metal-contaminated environments with free metal concentrations in the millimolar range (1−5 mM Zn(II), similar to the relatively high Zn(II) exposure in this study).[24] Intracellular sequestration generally occurs through metallothionines, and this type of resistance has been described to be induced by Cd(II), Zn(II), and Cu(II).[39] Furthermore, intracellular sensing of Zn(II), with subsequent gene regulation, has been reported for *B. subtilis* where metalloregulatory proteins control the intracellular concentration of free Zn(II) and allow for stepwise activation of different responses at different metal concentrations.[40] These examples illustrate that the responses monitored at the bacterial surface using XPS are not only related to pure adsorption/desorption and/or metal precipitation but that biological processes triggered by the presence of Zn(II) may play a large role in the surface chemistry of the bacteria. As an example of this, Mirimanoff et al. showed that the uptake of Zn(II) by Gram-positive bacterium could only be explained if both chemical adsorption mechanisms and biological resistance mechanisms were taken into account.[14] The changes in C 1s spectra observed after Zn(II) exposure, in this study, are thus reflections of biological alterations of the bacterial surface which can be hypothesized as up-regulation of efflux pumps,[39] leading to higher levels of peptide at the surface. However, it is likely that also other response mechanisms were triggered in the bacterium and could be traced at the bacterial surface. This illustrates the complexity of the bacterial surface chemistry in response to external stimuli such as metal exposure or different pH values. Furthermore, this work shows that an increased understanding of how bacterial surfaces respond can be obtained using surface sensitive analysis techniques such as cryo-XPS.

CONCLUSION

In the present study we have shown that the surface composition of bacterial cells changes throughout the pH range studied and that the presence of metal ions further influences the chemical composition of the cell wall. We have also confirmed previous studies showing that *Bacillus subtilis* accumulate Zn(II) at the surface when exposed to Zn(II) in solution. These findings have implication for how models predicting the adsorption of metals and protons to bacterial surfaces are constructed, interpreted, and understood. Furthermore, we have shown that by using cryo-XPS, we can obtain important surface compositional information that can assist in understanding molecular level processes at the bacterial surfaces as well as constructing more complex adsorption models. In this study we used *B. subtilis* bacteria, but the method could be applied to study surface alterations of any type of bacterial sample in more or less any type of conditions. The strength of using cryo-XPS in connection with the multivariate data treatment model is that it presents a way to reduce sample preparation and still obtain important biochemical information about the bacterial surface. Furthermore, the results obtained using this method are comparable to previously published XPS data treatment methods enabling comparisons to be made with literature data. This work, thus, shows that XPS is an important tool that can enable us to better understand the role that bacteria play in the biogeochemical cycling of metals and how their surfaces are affected by these processes.

ASSOCIATED CONTENT

Supporting Information

Fit between data and multivariate model in C 1s spectra for samples in Figures 4a and 6a. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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