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Surface characterisation of *Escherichia coli* under various conditions by near-ambient pressure XPS

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Bacteria are inherently in a hydrated state and therefore not compatible to ultra-high vacuum techniques such as XPS without prior sample preparation involving freeze drying or fast freezing. This has changed with the development of near-ambient pressure (NAP)-XPS, which makes it possible to characterise the bacterial surface with minimal sample preparation. This paper presents NAP-XPS measurements of *Escherichia coli* under various NAP conditions: at 11 mbar in a humid environment, at 2 mbar after drying in the chamber, pre-dried at 4 mbar, and at 1 mbar after overnight pumping at 10^{-4} mbar. The high-resolution spectra of carbon, nitrogen, and oxygen are presented and found to be in general agreement with XPS measurements from freeze-dried and fast-frozen bacteria. However, it was found that the amount of carbon components associated with polysaccharides increases relative to aliphatic carbon during drying and increases further after overnight pumping. This implies that drying has an impact on the bacterial surface.

KEYWORDS
bacteria, *E. coli*, NAP-XPS

1 | INTRODUCTION

XPS provides elemental and chemical information from the outermost approximately 10 nm of the sample surface. This is the same order of magnitude as the thickness of the outer bacterial membrane of gram-negative bacteria, as well as outer membrane molecules as exopolysaccharides and lipopolysaccharides, commonly attached to the cell surface.[1] The most apparent obstacle when studying biological samples with XPS is that bacteria are inherently in a hydrated state, while XPS is an ultra-high vacuum technique. To make bacterial samples compatible to ultra-high vacuum, generally 2 methods are used: freeze drying and fast freezing.[2,3] Both methods rely on separating bacteria from the liquid growth medium by washing and centrifugation. The resulting pellet is then either freeze-dried and crushed into a powder or fast-frozen in liquid nitrogen and kept frozen during measurements (Cryo-XPS). Freeze drying is believed to alter the bacterial sample, especially for gram-negative bacteria, which have a more fragile cell membrane than gram-positive bacteria.[4-6] Fast freezing preserves the water within the bacteria and is therefore conserving the bacterial structure better than freeze drying.

While there are many examples where near-ambient pressure (NAP) XPS has been used to study electrochemical processes and heterogeneous catalysis,[7,8] little attention has been paid to its potential use in biology-related fields. Model biomolecules, e.g., self assembled thiol monolayers functionalised with lipids, have been studied with NAP-XPS,[9] but to the best of our knowledge, bacteria have previously not been characterised by NAP-XPS. With this method, bacteria and biofilms on substrates can be characterised in different gas environments with minimal sample preparation. This decreases the possibility of introducing artefacts during sample preparation, allows for in situ measurements and studies of biofilm-substrate interactions.

In this paper, we present measurements of *Escherichia coli* (*E. coli*) adhered to silicon wafers under various conditions: in humid atmosphere at 11 mbar, after drying in the measurement chamber at 2 mbar, pre-dried at 4 mbar, and after overnight pumping at 10^{-4} mbar. The XPS spectra from nitrogen, carbon, and oxygen are presented, and changes in the spectra acquired under different conditions are discussed.

2 | EXPERIMENTAL

E. coli K-12 was obtained from the University of Nottingham, UK. This strain was routinely grown aerobically at 37° C on LB-agar plates[10]; liquid cultures were grown while shaking at 140 rpm. For bacterial adhesion, an LB-medium overnight culture was prepared. LB-medium of 5 mL was inoculated with single colonies from a freshly grown LB-agar plate and incubated overnight at 37° C and 140 rpm. Then, 1 mL of the culture was centrifuged (2 min, 3300*g*) and resuspended in 1 mL M9 medium (including 10 mM glucose). This cell suspension was diluted to an optical density at 600 nm (OD600) of 0.05 with M9 medium.

Silicon wafers were placed separately into wells of a sterile 6-well multiplate (Thermo Fisher Scientific, USA), and 5 mL of the diluted cell suspension was added. After an initial sedimentation phase of 1 hour without shaking, the samples were incubated at 37° C while shaking at 60 rpm. After 24 hours, the bacterial suspension was replaced by phosphate buffered saline (PBS), containing 8 g/L NaCl, 0.2 g/L KCl, 1.44 g/L Na_2HPO_4, and 0.24 g/L KH_2PO_4 with pH 7.4. After 2 minutes of immersion, half of the PBS volume was replaced by fresh PBS and again immersed for 2 minutes. This procedure was repeated twice to remove all nonadhering bacterial cells.

Measurements were conducted with the laboratory NAP-XPS instrument EnviroESCA at SPECS' demonstration lab (Berlin, Germany), equipped with a monochromated Al K_α radiation source and a differentially pumped energy analyser connected to an exchangeable sample environment. A more detailed description of the experimental set-up can be found elsewhere.[11] Two *E. coli* samples were characterised, both at 2 different pressures. One sample was immediately transferred to the load-lock chamber after being rinsed with PBS and measured in humidity at 11 mbar, the condition where the bacteria is closest to its natural, hydrated state. It was then gradually dried in the chamber while pumping, and measured at 2 mbar. The second sample was air dried before it was introduced into the measurement chamber and measured first at 4-mbar air atmosphere, then at 1 mbar after overnight pumping in 10^{-4} mbar, to examine if the dry state influences the *E. coli* XPS spectra.

All core level spectra were acquired with a pass energy of 50 eV, a step size of 0.1 eV and a dwell time of 0.25 seconds. The residual energy shift of the binding energy scale after environmental charge compensation by the gas was corrected for all spectra with respect to the emission line of aliphatic carbon at 285 eV. Peak fitting was conducted with Unifit 2018.[12] Generally, full width at half maximum (FWHM) was set as a free parameter but constrained to be the same for all peaks within the same spectrum. This did not apply to the core level peaks originating from gas, which inherently have a different peak shape and FWHM. In some cases, the peak position was fixed based on the peak position of the same peak acquired at 2 mbar for the C1s and O1s peak and at 1 mbar for the N1s peak. Details on peak positions, FWHM, charge corrections, intensities, and constraints are listed in Tables S1 to S3.

After measurements, the samples were characterised with a Zeiss Supra 40 scanning electron microscope (SEM), equipped with a Schottky field-emitter high-resolution cathode and an In-Lens detector. For elemental analysis, an EDX spectrometer with a UltraDry SSD detector (Thermo Fisher Scientific, USA) was used.

3 | RESULTS

XPS data from freeze-dried bacteria have commonly been analysed by calculating atomic ratios and relating them to cell constituents based on reference measurements and chemical composition, resulting in a set of equations that estimate the relative amount of polysaccharides, peptides, and hydrocarbons.[13] This method is generally not suited for NAP-XPS, nor Cryo-XPS, since the presence of water influences the chemical composition. To overcome this, relating the spectra to model compounds by principle method analysis has been proven a useful approach.[14] However, for these initial measurements, data analysis is limited to peak fitting and assessment of how peak area ratios of components vary with conditions.

By peak fitting, the carbon, oxygen, and nitrogen 1s core level peaks can be assigned to organic components originating from polysaccharides, lipids, and proteins/peptides. This is in accordance with previous findings from freeze-dried and fast-frozen bacteria.[2,6,15,16] In addition, phosphorous was detected in all cases except at 11 mbar, where the concentration was below the detection limit. Sodium, potassium, and chloride were also detected, which are the constituents of PBS.

Figure 1A displays the C 1s high-resolution spectra under NAP conditions, which were fitted with 4 components. The spectra are normalised to the maximum intensity of C1. The first component (C1) is attributed to aliphatic carbon at 285 eV; C2 is attributed to single bounded carbon from polysaccharides or amide groups ($C-O,\ C-N$); C3 originates from carbon located in carbonyl ($C=O$), amide ($O=C-N$), and acetal ($O-C-O$) groups. C4 is assigned to carbon from carboxyl and ester groups ($[C=O]-O-R, R=H, C$). A comparison of the component areas reveals that C2, C3, and C4 increase relatively to aliphatic carbon as the pressure decreases. After overnight drying, the C1 and C2 components are approximately equally large in terms of peak areas, and the peak from carboxylic carbon (C4) is at its largest value, while not being detected at all at 11 mbar. Note that the signal-to-noise ratio is lower at 11 mbar compared to the other conditions, due to increased electron scattering in the gas atmosphere at higher pressure and therefore overall lower count-rate. Details on the peak area ratios for carbon, oxygen and nitrogen are listed in Table S4 to S6 in the supplementary information.

Figure 1b displays the N 1s spectra under various conditions. All peaks are normalised to maximum intensity of component N1, except of the inset at 4 mbar showing the complete spectrum. The first component at 400.1 eV originates from amide ($[C=O]-N$), and possibly also amines (NH_2), which are difficult to distinguish based on difference in binding energy. The spectra from the measurements at 4, 2, and 1 mbar also show a small peak shifted 1.1 eV towards higher binding energy, which is assigned to a protonation of the amino groups commonly occurring on the bacterial surface.[2] The nitrogen gas peak (N3) is not detected at 11 mbar, because water vapour, and not air, is the dominating gas in vicinity to the sample surface. The peak area after correction for the spectrometer transmission function, electron inelastic mean free path and Scofield photoionisation cross section allows for comparison of atomic ratios between elements. These reveal that the nitrogen amount relative to the amount of total carbon is approximately 0.13 for all conditions. This is close to N/C ratios calculated from

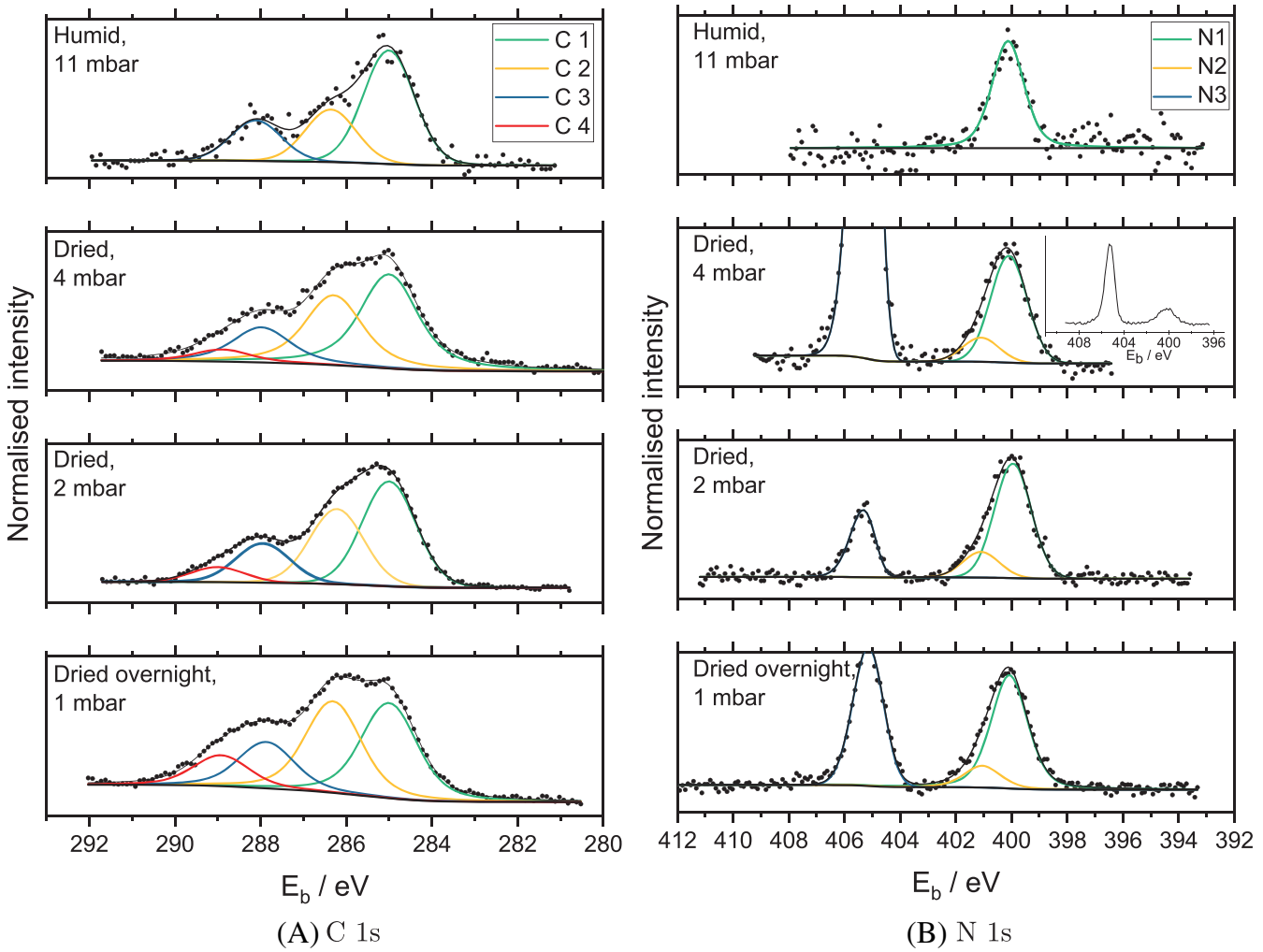

FIGURE 1 The XPS spectra for A, carbon and B, nitrogen acquired under 4 different conditions. The component peaks are explained in the text. The inset shows the complete N1s spectrum of dried sample at 4 mbar

various freeze-dried *E. coli* samples, with reported values varying from 0.026 to 0.132.[6] For details, see Table S5.

The O 1s high-resolution spectra in Figure 2 show 2 groups of main features: 1 from the sample (at lower E_b) and 1 from ambient gas (at higher E_b). All spectra were normalised to the maximum of the sample feature. Component O1 at 531.1 eV is assigned to double-bounded oxygen in carboxyl, amide, and phosphate groups. O2 is shifted approximately 1 eV from the first component and originates from single bounded oxygen from hydroxyl $(C-OH)$ and acetal groups $(C-O-C)$. O3 is shifted 2.1 eV from O1 and assigned to single-bounded oxygen from ester and carboxyl groups $([C=O])-\underline{O}-R, R=C, H)$, as well as oxygen from adsorbed water.[17,18]

Component O4 and O5 originate from oxygen gas species in vicinity of the sample surface, mainly water vapour.[19] A contribution from Na KLL Auger peak at approximately 535.6 eV can also not be excluded. The gas species cannot be referenced to the sample Fermi level, which means that charge correction with respect to the sample does not necessarily apply for the gas species. This explains the observed shift towards higher binding energies for gas species at 4 and 1 mbar.

The peak assignment for oxygen is consistent with literature,[2] however, when it comes to interpretation, it must be done with great care. The oxygen peak is generally broad, and the sample contains a variety of oxygen species that are close in binding energy. When drying, water evaporates from the sample, and oxygen from phosphate in the buffer solution cannot be distinguished from oxygen from amide or carboxyl groups. Considering these complicating factors, the carbon and nitrogen spectra provide more reliable information.

Representative SEM micrographs of the investigated samples are shown in Figure 3. In addition to the rod-shape bacteria, residues from PBS buffer solution appear as bright salt crystals in Figure 3A. The bacteria seem to be partially capped in salts (therefore the bright contrast), with black heads at the end where the bacteria are not covered by salt. This view is supported by EDX-spectra revealing the elemental composition of the various features, which can be found in the Supporting Information. The bacteria seem to be damaged, and it is likely that cell material is expelled from the cells due to shrinkage or capillary forces during drying. The sample shown in Figure 3B was prepared following the same protocol but rinsed with DI water. In addition to no salt residues, the cells seem to have a more even shape. Although buffer solutions make sense from a biological perspective, rinsing with DI water should be evaluated as an alternative potentially causing less cell damage during drying and not leaving residues on the samples. Rupture of cells during drying is likely to expose the lipid-rich cell membrane compared with intact cells. This would then result in an increased car-

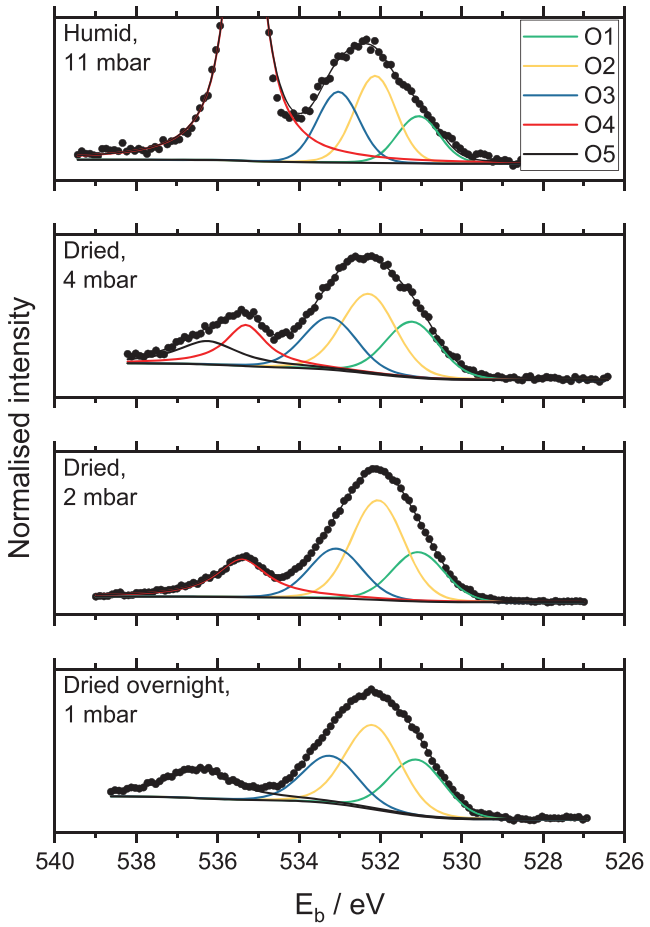

FIGURE 2 The O 1s XPS spectra acquired under 4 different conditions. The component peaks O1 to O5 are explained in the text below

boxyl peak (from the polar head of the lipid), and also a strong increase of aliphatic carbon (from the unpolar tail). Our measurements show an increase in carboxylic carbon during drying; however, it is not accompanied by an increase in aliphatic carbon.

4 | DISCUSSION

Bacterial properties vary greatly with factors as bacterial type, substrate, growth medium, and pH.[4,16] While the sample is still hydrated, water and salts from the buffer solution influence the surface properties, and this changes with drying. As the bacteria dry, they shrink, which means that more of the outer cell membrane comes within reach of the XPS information depth of approximately 10 nm. Structural changes on the bacterial surface due to change in pH and hydrophobicity may also occur. Lipopolysaccharides are found on the outer membrane of gram-negative bacteria. These are anchored to the membrane by lipid-A with long chained polysaccharides protruding from the cell membrane.[1] Lipopolysaccharides for *E coli* strain K-12 have been determined to be 3 ± 2 nm long.[20] The polar head of lipid-A contains carboxyl groups, while polysaccharides contain hydroxyl, acetal, and carboxyl groups (identified as C2, C3, and C4 in Figure 1A). Consequently, a reason for the changes in the carbon spectra during drying can be that more of the lipopolysaccharides, and other polysaccharide-based surface molecules are detected, either due to drying, reorganization, cell damage, or a combination thereof. Ramstedt et al[16] did a study on how the carbon spectrum changed after sublimating the water from fast-frozen bacteria. Relative to fast-frozen bacteria, an increase in lipid content and decrease in protein content were found after drying. The results cannot be compared directly, as this was done with the gram-positive bacteria *Bacillus subtilis*. However, both cases show that drying causes substantial changes on the bacterial surface. Obviously, there is a need to develop experimental strategies to hold the bacteria in a humid environment to get valid XPS spectra. In our study, spectra acquired at 11 mbar water pressure seem to be the most realistic ones in terms of "real life" conditions of such bacterial samples.

The sample inevitably contains carbon contamination, which is indistinguishable from carbon from bacteria. However, as long as the contamination level is constant at all conditions, this is a systematic error that does not affect the spectral trends. Further, beam damage also needs to be considered. Beam damage is indicated by a decrease in carbon bound to oxygen or nitrogen, and an increase in aliphatic carbon during measurements.[13] This is the opposite of the trend observed from our data. In addition, generally no notable changes were observed in the carbon spectra between the first and the last sweep of a measurement.

Silicon wafers were chosen as a model substrate, as it is easily available and electrical conductive. However, various substrates as for instance glass, steel, titanium, and other biomedical-relevant materials can be used. Thus, bacteria and biofilms can be grown directly on the chosen substrate under controlled conditions, and studied in near-ambient conditions with minimal alteration of the sample. Near-ambient pressure XPS can thereby provide new insight about the

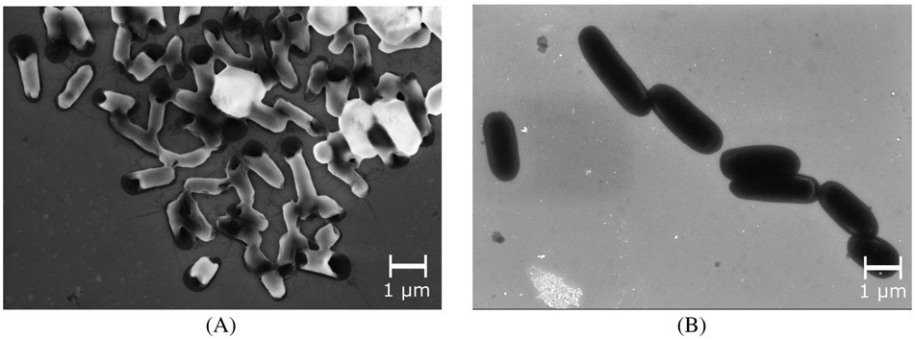

FIGURE 3 Representative SEM-micrographs of *E. coli* on Si wafers. Sample A was rinsed with PBS buffer, while sample B was rinsed with DI water

bacterial surface and bacteria-substrate interface, for instance, concerning microbial-induced corrosion and biofilm growth on medical implants.

5 | CONCLUSIONS

The surface of *E. coli* adhered to a Si wafer has been characterised by NAP-XPS from a humid to a dry state. The information from the core level peaks of oxygen, carbon, and nitrogen makes it possible to identify components originating from polysaccharides, lipids, and proteins/peptides, in accordance with previous findings from freeze-dried and fast-frozen bacteria. Changes in the carbon spectrum were observed during drying and after overnight pumping at 10^{-4} mbar, suggesting that drying causes substantial changes on the bacterial surface.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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