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On-Cell MAS NMR: Physiological Clues from Living Cells

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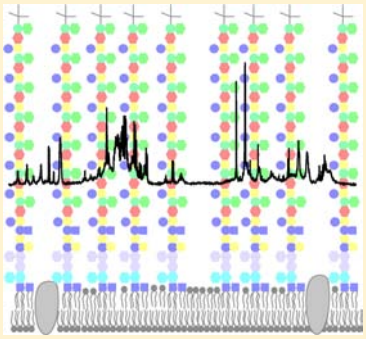
Supporting Information

ABSTRACT: While structural information on biomolecules is mainly obtained from purified in vitro samples, NMR can also be applied in the context of entire cells or organisms. The present study describes maturation processes in living *Salmonella enterica* serovar Typhimurium, a prevalent cause for human gastroenteritis. In our physiological study, we follow the composition of the O-antigen on the outer bacterial membrane with high-resolution MAS NMR spectroscopy. We detect and characterize an evolution of the O-antigen composition, in particular of the O-acetylation state of the O-antigen, a factor that can play an important role in vaccine development.



INTRODUCTION

Most of the atomic-resolution structural information available about biomolecules is obtained from in vitro X-ray or NMR investigations, where the molecule studied has been isolated, purified, possibly crystallized or prepared in a defined artificial medium, in an attempt to make it amenable to structural analysis while mimicking as much as possible its original natural environment. This reductionist approach remains a powerful and important methodology. There are situations, however, where molecular studies can be performed in the context of living cells. In-cell investigations of biomolecules, usually dissolved in the cytosol, or on-cell studies of highly dynamic species on the cell surface provide information about structure and dynamics under more physiological conditions. NMR is well suited for such studies. When the spectral line-width is narrow enough, liquid-state NMR methods can be directly applied to bacterial and eukaryotic cells.[1−7] For more rigid cell components magic-angle sample spinning (MAS)[8] becomes necessary to obtain high-resolution spectra. For instance, intact bacterial cells[9] and even whole nematodes[10] and crustaceans[11] could be investigated by MAS NMR. For these studies, the use of high-resolution MAS (HR-MAS)[12] probes turned out to be useful as they address both the need to spin the sample at the magic angle and to use high-resolution techniques as a ^{2}H-lock channel, pulsed field gradients, and a B_0-field homogeneity that allows for efficient water-suppression schemes. HR-MAS has been also successfully employed in the investigation of lipopolysaccharides (LPS) directly on bacterial cells.[13−16] In Gram-negative bacteria LPS is the major component of the outer membrane. It consists of the Lipid A, a core structure, and the O-antigen, a polysaccharide chain constituted of up to >100 repeats of an oligosaccharide.[17] LPS contributes to the stability of the outer membrane, serves as a barrier against detergents and antibiotics, protects the bacterium against the host immune reaction, and can also play an important role in the virulence of pathogenic bacteria.[18] HR-MAS NMR spectroscopy is a powerful tool to investigate the O-antigen on the intact cells as the O-antigen leads to intense and relatively narrow signals. Because of the extracellular nature of the LPS, the corresponding experiments have been termed "on-cell" NMR.[2]

Bacterial growth in laboratory batch cultures can be distinguished by four consecutive growth phases: lag phase, exponential growth phase, stationary phase (where the number of cells stays roughly constant due to nutrient depletion and accumulation of waste), and death phase. Previous on-cell NMR studies[2,13−16] aimed at clarifying the composition of LPS and capsular polysaccharides in cells in the stationary or late stationary growth phase. In the following, we show that the composition of the O-antigen changes over time even in the stationary phase, as the O-acetylation state changes. This is important because the presence and position of *O*-acetyl groups in the O-antigen chain, e.g., in *Salmonella enterica* subspecies 1 serovar Typhimurium (*S.* Typhimurium) or *Shigella flexneri* determine the serotype of a certain strain and therefore play a key role in bacteriological analysis of complex samples by typing sera. In addition, *O*-acetyl groups are important in vaccine development.[19−22]

We detect this maturation by on-cell HR-MAS NMR in living *S.* Typhimurium cells.[23] The O-antigen of this subspecies consists of 70−100 repeating [→2)-α-D-Man*p*-(1→4)-α-L-Rha*p*(1→3)-α-D-Gal*p*(1→] units, where Rha stands for rhamnose, Gal for galactose and Man for mannose (Chart 1). The Man residue is substituted at carbon C3 with α-linked abequose (Abe; 3,6-dideoxy-D-xylo-hexose), O-acetylated at

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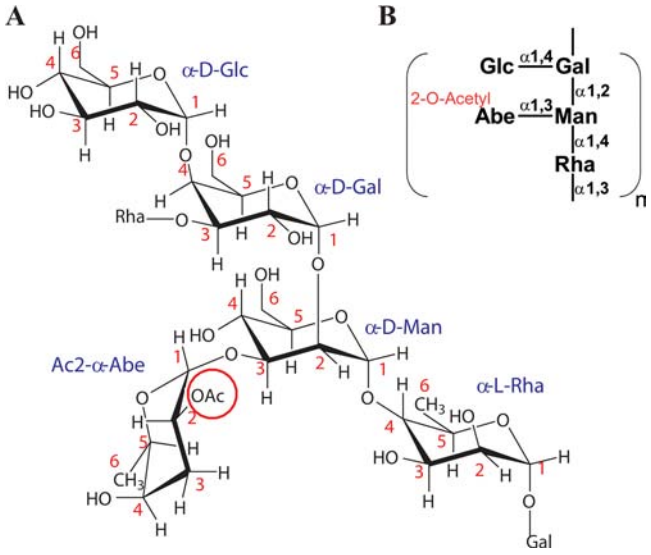
Chart 1. A: Chemical Structure with the *O*-Acetyl Group at Abe Marked by a Red Circle. B: Simplified Notation



C2. The Gal residue is nonstoichiometrically substituted with a glucose (Glc) residue, either in α-(1→6) or α-(1→4) linkage.[24−26]

EXPERIMENTAL SECTION

Preparation of *S.* Typhimurium Cells. The *S.* Typhimurium strains used were either the histidine auxotroph wild-type strain SL1344[27] or a strain without functional acetyltransferase gene (M2012: SL1344 *oafA*::Tn10; from A. Sturm, Institute of Microbiology, ETH Zurich.) Bacteria were grown in Luria−Bertani (LB) medium (10 g/L Bacto tryptone, 5 g/L Bacto yeast extract, 10 g/L NaCl). LB agar plates were supplemented with 1.5% (w/v) agar. Streptomycin was added to a final concentration of 50 μg/mL. *S.* Typhimurium cells were grown for the stated time and, at the given time points, the equivalent of 12−15 OD_{600} (optical density at 600 nm) was pelleted by centrifugation for 10 min at 4 °C and 3750 g. The pellet was resuspended in 10 mM Potassium Phosphate Buffer (PPB; 10 mM pH 7.4) in D_2O (or H_2O where stated) to a final concentration of about 0.2 OD_{600}/μL in PPB containing acetone (final concentration 0.1% (v/v)) as internal reference. We have verified a certain variability in the amount of cells in the rotor, likely due to the centrifugation step in the sample preparation procedure. The sample corresponding to Figure 2A−C was prepared in a similar way, except that the pellet was resuspended in its own supernatant and 10% D_2O containing acetone was added. For the experiments showing the influence of pH cells were grown for the stated time in the original or the pH-adjusted medium and, after further growth, the equivalent of 12−15 OD_{600} was pelleted as described above. The pellet was resuspended in 10% NaN_3 in 10 mM PPB in D_2O and incubated at room temperature for 1h. The cells were washed once with PPB and to a final concentration of 0.2 OD_{600}/μL in PPB containing acetone (final concentration 0.1% (v/v)) as internal reference. For the experiments showing the influence of O_2 on the cell growth cells were grown for the stated time in LB medium under an N_2-atmosphere. At the given time points, the equivalent of 12 OD_{600} was pelleted as described above. The pellet was resuspended in 10% NaN_3 in 10 mM PPB in D_2O and incubated at room temperature for 1h. The cells were washed four times with PPB and resuspended to a final concentration of 0.2 OD_{600}/μL in PPB containing acetone (final concentration 0.1% (v/v)) as internal reference.

NMR Measurements. ^{1}H NMR experiments were carried out on a Bruker Biospin AVANCE II+ spectrometer operating at 850 MHz ^{1}H Larmor frequency using a 4 mm HR-MAS Bruker probe with 50 μL restricted volume rotors at a temperature of 29 °C and a MAS frequency of 4 kHz. The 1D experiments were performed with the Carr−Purcell−Meiboom−Gill (CPMG) pulse-sequence (90° pulse-(τ −180° pulse-τ)$_n$-acquisition)[28] to remove broad lines due to solid-like material. The CPMG pulse (n*2τ) was 20 ms with τ equal to the spinning period. The 90° pulse was set to 9 μs, the spectral width to 15 ppm, the acquisition time was 1.28 s and number of scans 400. The signal of HDO was attenuated using water presaturation for 3 s. 2D NOESY spectra were recorded in TPPI mode acquiring 48 scans for each of the 460 t_1 increments and 2048 increments in t_2. Data were apodized in both dimensions with squared sine multiplication with 90° phase. In the t_1 dimension a linear prediction to 2048 experiments was applied.

Results and Discussion. The ^{1}H on-cell HR-MAS NMR spectra of living *S.* Typhimurium cells grown to exponential phase (4 h growth time, OD_{600} = 1.25) are shown in Figure 1.

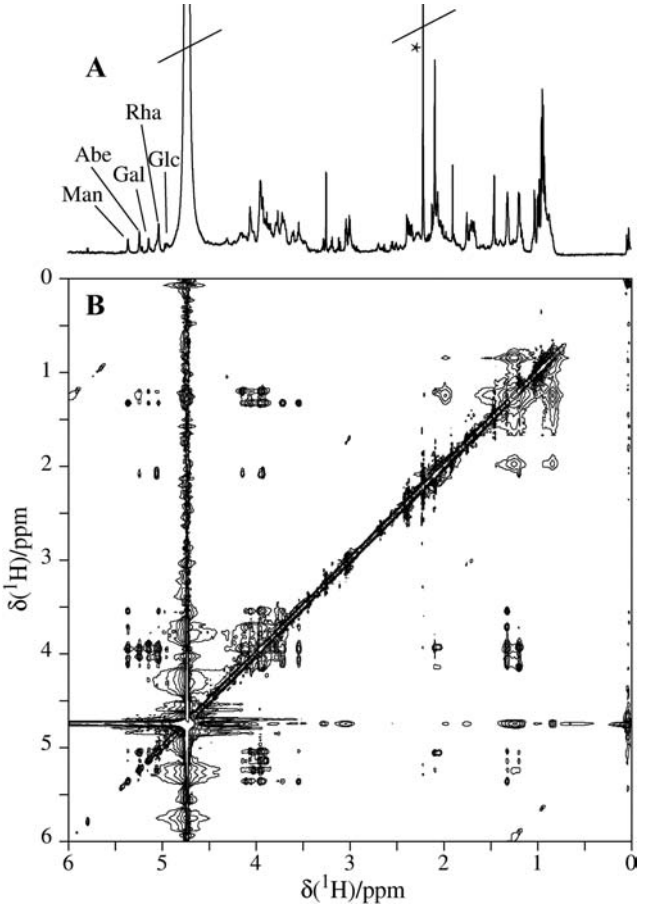


Figure 1. A: ^{1}H HR-MAS NMR spectra of living cells of *S.* Typhimurium in the exponential phase (4 h) with the assignment of the O-antigen anomeric protons (H1) signals. The intense peaks which have been cut correspond to HDO and acetone, the latter (denoted by an asterisk) added as internal chemical-shift reference. B: NOESY spectrum with mixing time of 120 ms.

The major signals were assigned by 2D experiments (e.g., the NOESY spectrum in Figure 1B) and by comparing with the ^{1}H chemical shifts reported in literature.[24] It is convenient that the signals of the protons at the anomeric carbon C1 (anomeric protons, H1) in the ^{1}H on-cell MAS NMR spectra appear in a spectral region (4.9−5.4 ppm) which is relatively free from peaks due to other cell components, or to soluble molecules, e. g. monomeric sugars (see Figure S1 in the Supporting Information, SI). This, together with the high intensity of the O-antigen signals, allowed us to investigate the O-antigen composition by analyzing the signals of the anomeric protons directly in the intact cells, thus avoiding the isolation of the LPS. Our analysis

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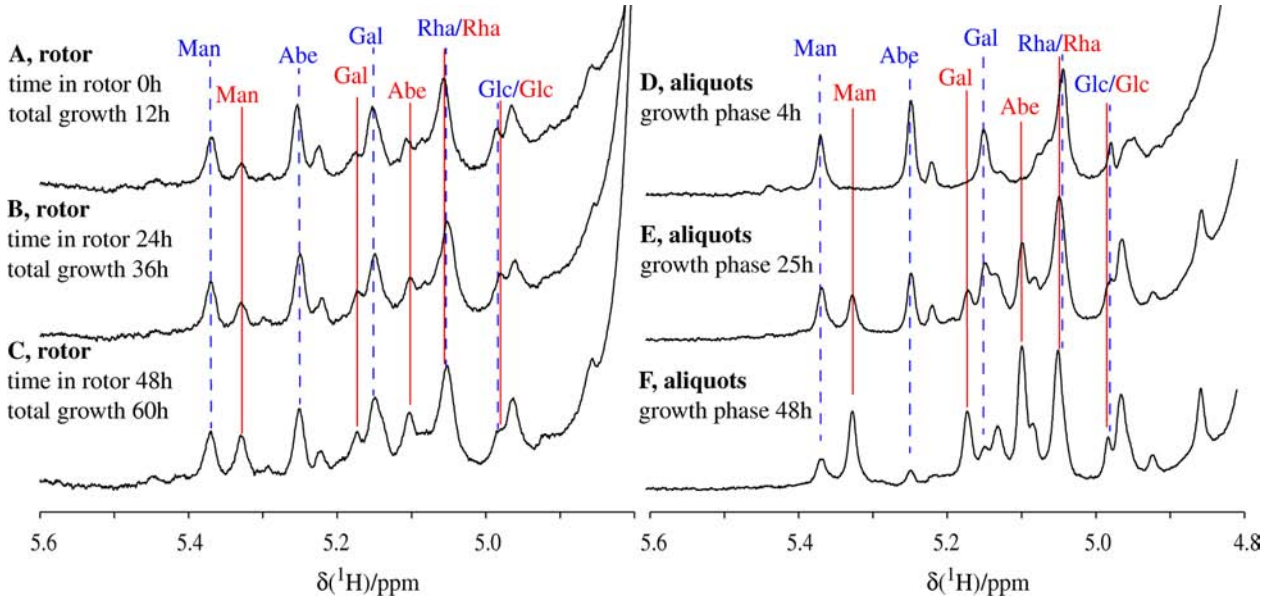

Figure 2. ^{1}H MAS NMR spectra (anomeric region) of living *S.* Typhimurium cells. Left panel: Cells from an early stationary phase culture (A: 12 h, OD_{600} = 4.31) were centrifuged and the pellet was resuspended in the supernatant. The same sample was then measured after further 24 h (B) and further 24 h (C) in the rotor at 37 °C. "Total growth" indicates the time interval equal to the proper growth phase in LB medium plus the time in the rotor. The corresponding values of the O-acetylation degree are reported in Figure S2 of the SI. Right panel: Cells from a culture in the exponential phase (D: 4 h, OD_{600} = 1.25), late (E: 25 h, OD_{600} = 3.05) and very late stationary phase (F: 48 h, OD_{600} = 2.74). The spectra correspond to three cell aliquots taken from the same culture at the given time points, centrifuged and then resuspended in buffer. The corresponding values of the O-acetylation degree are reported in Figure S5 of the SI, together with data from additional experiments. The assignment of the anomeric protons of the O-antigen is reported, the blue broken line and the red line correspond to the O-antigen which is O-acetylated and de-O-acetylated at Abe C2, respectively. Figure S3 of the SI shows the spectral region at about 2 ppm of the spectra here reported, qualitatively confirming the evolution of the intensity of the $-COCH_3$ signal.

may miss some O-antigen repeats close to the cell surface which may be solid enough to be filtered out by the CPMG pulse sequence. The assignment of the O-antigen proton signals is reported in Table S1 (SI) and is indicated for the anomeric protons in Figure 1A. The ^{1}H on-cell NMR peak integrals of the H1 of Abe, Man, Gal, and Rha are comparable (Table S1 of the SI), in accordance with the repeat unit in Chart 1.[24−26] The H1 peak of Glc at 4.98 ppm has a roughly 10 times smaller integral, indicating that only about 10% of the O-antigen repeats are glucosylated at the Gal residue.[29] Finally, Abe is practically entirely O-acetylated in this growth phase, since no resonances from de-O-acetylated Abe can be detected in the spectrum.

In a next step, the evolution of the NMR spectra during the stationary growth phase was followed using a single sample of living *S.* Typhimurium cells. The sample was concentrated from a bacterial culture in the early stationary phase (12 h, OD_{600} = 4.31) by centrifugation, resuspended in sterilized spent growth medium, and transferred to the NMR rotor. Spectra were recorded on three consecutive days leaving the cells in MAS rotor during the whole time. Spectra are given in Figure 2, left panel. Directly after filling the NMR rotor with bacteria that were grown 12 h to reach the early stationary phase the main resonances are identical to the ones of cells grown to the exponential phase (4 h) in Figure 1A but an additional, weaker set of signals becomes visible (red lines in Figure 2). The spectra measured 24 h later (Figure 2B) and after further 24 h (Figure 2C) witness a significant growth of these additional signals. These new peaks correspond to a modified O-antigen repeat which is de-O-acetylated at Abe C2 position (Chart 1) as suggested by the ^{1}H chemical shifts of the Abe ring. In fact, the H2 peak appears at higher fields (4.03 ppm) due to the absence of the deshielding *O*-acetyl group at C2 (the chemical shift of H2 in O-acetylated Abe is 5.06 ppm). The absence of the *O*-acetyl group at Abe C2 also resulted in significant shifts for other residues (−0.04 ppm for Man H1 and +0.02 ppm for Gal H1). The assignment of the ^{1}H signals of de-O-acetylated O-antigen is reported in Table S1 of the SI. The fraction of O-acetylated O-antigen decreases from about 75% in the spectrum A to about 55% in spectrum C (Figure S2 of the SI). The decrease in the amount of O-acetylated O-antigen is qualitatively confirmed by the reduction of the intensity of the $-COCH_3$ peak (Figure S3A−C of the SI). An analogous experiment where cells from a culture in the exponential phase (4 h) were investigated, gave similar results, namely the appearing of signals due to O-antigen de-O-acetylated at Abe C2 (Figure S4 of the SI).

To demonstrate that the MAS environment does not decrease cell viability, we determined the number of colony-forming units (cfu),[1,5] a convenient proxy for the viability of bacterial cells. The cfu was roughly the same before and after an NMR experiment of 1 h showing that most of the bacteria survived the centrifugal acceleration up to about 10^5 g (8 kHz in a rotor with 4 mm external diameter) and a certain O_2 depletion during the measurement period inside the sealed rotor (Figure S6 of the SI). It should be noted that between the NMR measurements of Figure 2A−C, we kept the NMR rotor open (thus allowing oxygenation) and the sample shaking at 37 °C in order to maintain the system in conditions as close as possible to the ones of the batch cell culture. An overnight experiment with a MAS frequency of 4 kHz did not significantly affect cell survival either (Figure S6 of the SI). Our data agree with literature reports on post-MAS cell viability. In fact, while mammalian cells were found to be fragile under MAS,[30−32] sample spinning had minimal effects on the survival of yeast[33] and bacterial cells.[34]

The results obtained from living cells maturing in the rotor were then compared to spectra of aliquots taken from a *S.* Typhimurium culture at different time points, corresponding to different growth phases (exponential phase shown in Figure 2D, late stationary and very late stationary phase in Figure 2E and F, respectively). Characterizing all of the aliquots, we noted that the pH of the growth medium increased at longer cell-growth durations, from 7.15 at 4 h to 8.92 at 48 h. To concentrate these samples for the NMR studies the cells were centrifuged, and resuspended in a neutral buffer using D_2O to aid solvent suppression. Comparing the spectra of the different aliquots from increasing growth phase (Figure 2D−F) showed a similar time

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evolution as for the cells that matured in the rotor (Figure 2A−C), namely the progressive appearance of a second set of signals (at the positions of the red line) corresponding to the O-antigen de-O-acetylated at Abe C2. This finding was confirmed by the analysis of the aliquots with an immunoblot developed with an antiserum able to recognize the *O*-acetyl group (Figure S7 of the SI). The fraction of O-acetylated O-antigen decreases from about 100% in the exponential growth phase to about 30% in the very late stationary phase as deduced from the integrals of the Man H1 NMR signals (Figure S5 of the SI).

We also observed that the O-acetylation state of the O-antigen of cells from a cell culture with a defined growth phase did not significantly change upon time (spectra in Figure S8 of the SI) after being resuspended in the buffered medium in a sealed rotor, in contrast to the O-acetylation of the O-antigen of the bacteria left in the (unbuffered) spent growth medium in the open rotor (Figure 2A−C). Nevertheless, the viability of the cells is about the same (the corresponding cfu are reported in Figure S6 of the SI). Buffering and keeping the rotor sealed is therefore beneficial for the measurement of multidimensional NMR spectra on living cells that require several hours of data acquisition.[35]

In order to investigate the relationship between the change in the O-acetylated state of the O-antigen and the pH change in the unbuffered growth medium during the bacterial growth (vide supra), we incubated *S.* Typhimurium cells in LB medium for 30 h and recorded the OD_{600} and pH (Figure 3A, B). The pH of the solution,

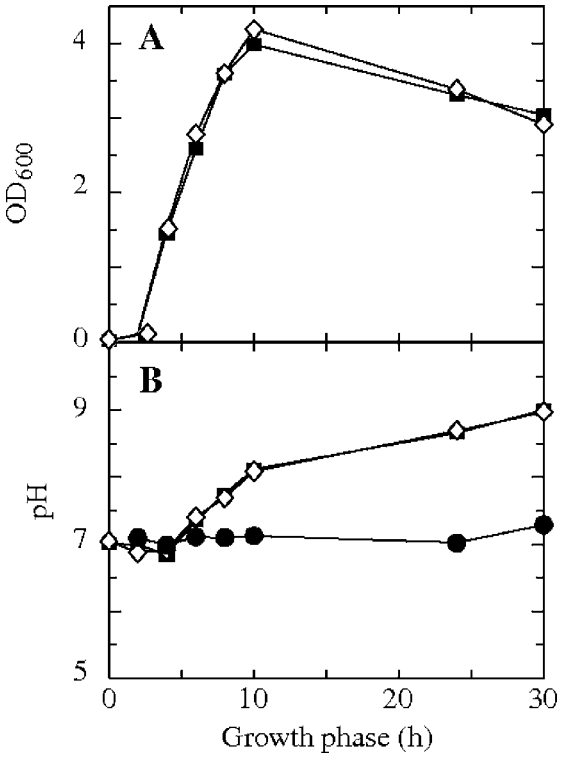

Figure 3. A: *S.* Typhimurium cells grown curve in LB medium. B: pH development during growth of *S.* Typhimurium. Full square symbols represent the SL1344 strain, empty diamonds M2012 strain and (only in B) full circles the pH in a LB solution without bacteria.

initially neutral, started to increase in the late exponential phase, until it reached a value of 9.0 after 30 h growth, in agreement with the pH changes observed for the points in time where the spectra of Figures 2D−F were recorded. We verified a similar behavior for *S.* Typhimurium M2012, a strain not expressing O-acetylated Abe. These findings suggest that the change in the O-acetylation state of the O-antigen at Abe C2 is correlated to the pH change of the growth medium as evidenced by the spectra of Figure 4.

To further investigate the relation between pH increase and de-O-acetylation, cells were grown to late exponential phase (8 h) reaching pH = 7.4 and OD_{600} = 3.85. At this point in time, the antigen is almost completely O-acetylated. The cell culture was then split into six samples. Samples A−C were grown for further 20 h in different media. Sample A was incubated in the same medium and the final pH was 8.7 (OD_{600} = 3.54). In sample B, the pH was adjusted to 5.2 and then incubated reaching a final pH of 6.8 (OD_{600} = 4.08). In C, the pH was adjusted to 8.8 with final pH of 9.0 (OD_{600} = 3.15). Samples F−H were resuspended in azide-containing potassium phosphate buffer at this time point to kill the cells (8 h). The pH in sample D was not changed artificially while the pH was modified in the other two: in sample E, pH was adjusted to 5.1 and in sample F to 9.4. All three samples were then also incubated for additional 20 h at room temperature. A repetition experiment confirmed the results of Figure 4.

We conclude that the change in the O-acetylation state occurs much faster at high pH values. Remarkably, the process was much faster for alive cells, thus suggesting that the process is more complex than a simple pH-catalyzed reaction.

As a further characterization, the role of oxygen in the context of the evolution of the O-acetylation state was investigated as we had noticed that the process observed within the rotor (Figure 2A−C) proceeded significantly slower than in the bacterial culture (Figure 2D−F). Such behavior could be due to the higher cell density and the reduced oxygenation in the NMR rotor. Therefore, we repeated the experiment corresponding to the spectra in Figure 2D−F with a culture grown under an N_2-atmosphere. The spectra of three different aliquots taken from identical cell cultures after 4 h, 24 h and 48 h cell growth are presented in Figure 5 and show no signals due to O-antigen de-O-acetylated at Abe C2 even at late cell growth phases. The pH of the cell cultures was about 8 at all times. These findings taken together suggest that (i) the evolution of the O-acetylation state of the O-antigen of *S.* Typhimurium is correlated to the change of the pH in the unbuffered growth medium during bacterial growth; (ii) such change of pH due to the cell metabolism takes place only in aerobic conditions.

■ CONCLUSIONS

On-cell MAS NMR is a well-established technique to investigate bacterial cell walls. In this study, we take advantage of on-cell MAS NMR to trace bacterial physiological processes at the molecular level in alive cells. Our on-cell in vivo NMR measurements show that the O-acetylation state of Abe C2 in *S.* Typhimurium evolves during bacterial growth. The alteration of the O-acetylation state only takes place in aerobic conditions; it starts in the early stationary phase and progresses with time. An increase of the pH of the growth medium was also observed and the two processes are correlated, namely high pH promotes the presence of de-O-acetylated Abe. We therefore see this evolution of the O-acetylation state of the O-antigen as a physiological process since the alteration of the pH of the growth medium is due to the presence of the living bacteria and the effect is much more pronounced in alive cells than in dead ones. This seems to indicate that *S.* Typhimurium bacteria might have an active role in this alteration of their O-antigen composition which could be advantageous for the bacteria: A change of the cell surface will most probably lead to different immunological properties of the cell and an altered immune response by the host.

The changes in the O-acetylation of the O-antigen can be of importance for the immune response to *S.* Typhimurium[36] but might also affect the infection process and the intracellular growth of these bacteria. It is known that in other species, e.g., *Neisseria meningitidis* or *Staphylococcus aureus*, the acetylation of cell surface capsular polysaccharides alters the immunological

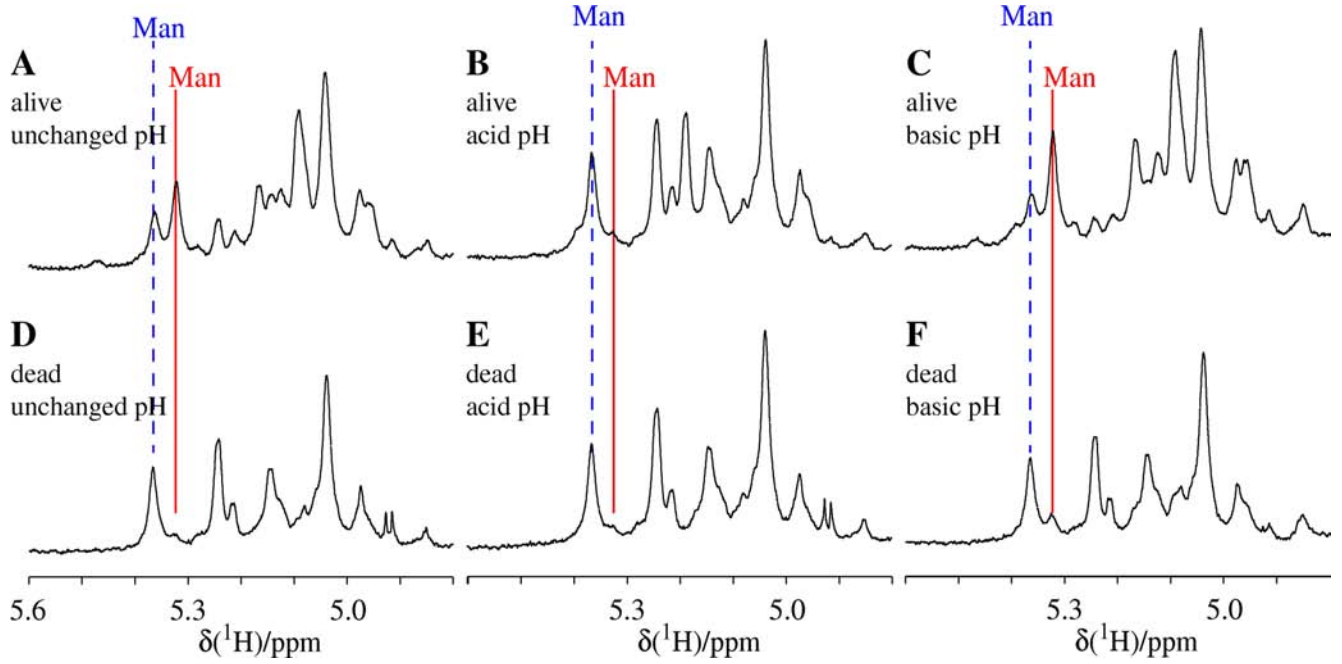

Figure 4. ^{1}H MAS NMR spectra of intact inactivated cells of *S.* Typhimurium in the late stationary phase (28 h). The assignment of the anomeric protons Man H1 of the O-antigen is reported, the blue broken line and the red line correspond to the O-antigen which is O-acetylated and de-O-acetylated at Abe C2, respectively. The upper row corresponds to alive cells whose medium was changed as indicated in the figure in late exponential phase and which were then further incubated for 20 h. The lower row corresponds to cells that were killed after 8 h under otherwise identical conditions, Figure S9 of the SI shows the region with the $-COCH_3$ signal of the spectra A–F.

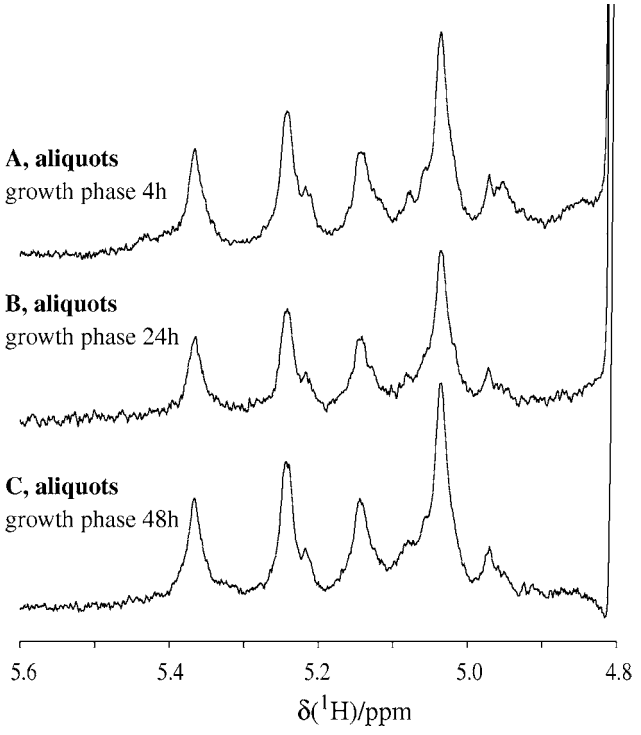

Figure 5. A: ^{1}H HR-MAS NMR spectra of intact cells of *S.* Typhimurium from a culture in the exponential phase (A: 4 h, OD_{600} = 0.66), late (B: 24 h, OD_{600} = 0.74) and very late stationary phase (C: 48 h, OD_{600} = 0.59) grown in anaerobic conditions. The spectra correspond to three cell aliquots taken from the three cultures at the given time points, centrifuged and then resuspended in buffer.

properties of the cells.[37−39] The influence of living cells on the pH of their environment and the evolution of the O-acetylation state of the O-antigen due to the alteration of pH appear to be controlled by complex mechanisms and more work is needed for a full understanding. We also note that the pH conditions where we observed an alteration of the O-acetylation state of the O-antigen of *S.* Thyphimurium infection occur in nature. For example, in the human intestine pH varies from 6.4 up to 8.2,[40] depending on diet and pathologies. In addition, we can also speculate that in most cases, a natural *S.* Typhimurium infection commences with an ingestion of bacteria from an aerobic environment. These bacteria will probably show a high amount of de-O-acetylated O-antigen. This would imply that *S.* Typhimurium cells' LPS first encountered by the host immune system will differ from the LPS of bacteria will be present in the host intestine and macrophages[41] after several rounds of replications under anaerobic conditions. This may therefore add an additional level of host immune system evasion by *S.* Typhimurium to its infection cycle.

The evolution of the O-acetylation state of the O-antigen was followed either on a single bacterial sample left at all times inside the NMR rotor or by studying aliquots from the same bacterial culture at different time points. The two methods offer distinct advantages. In fact, the NMR measurements on cells kept all the time in the NMR rotor allow to investigate the very same system continuously and potentially fully automated. The investigation of several aliquots from the same cell cultures consents to employ different analytical techniques on the same cell culture, thus providing complementary information on the system. It is interesting to note that the development of the bacteria in the rotor proceeds at a slower rate, but does not qualitatively deviate from the development in cell cultures.

Finally, we notice that the evolution in the O-acetylation state of the O-antigen caused by the spontaneous rise of the pH in aerobic conditions due to the bacterial metabolism and thus, the influence of the cell growth phase on the O-acetylation state

of the O-antigen may be a commonly occurring phenomena in bacterial cultures grown in the laboratory. Such phenomena should be carefully taken into account while determining the composition of the O-antigen. For example, incomplete O-acetylation was reported for capsular polysaccharides of *Neisseria meningitidis*[13,42] and O-antigens of *Hafnia alvei*[43] and *Shigella flexneri*.[20,21] As glycoconjugates containing polysaccharides are an interesting vaccine antigen[44,45] our findings may have a direct impact on vaccine development where O-acetylated structures are involved.

The results presented in this study hint at a new avenue where HR-MAS NMR can characterize physiological processes occurring on the cell surface.

■ ASSOCIATED CONTENT

Supporting Information

Additional data on the NMR spectra of the aliquots taken from a culture at two different time points compared to the spectrum of the supernatant solution; the O-acetylation state relative to the experiment corresponding to Figure 2A-C; the spectral region 2.2−2.0 ppm with the $COCH_3$ signal of the spectra in Figure 2; the NMR spectra relative to an "in rotor" experiment similar to the one in Figure 2A−C on cells from the exponential phase (4 h); the O-acetylation state of cell aliquots from cultures at different growth phase (Figure 2D−F); the cell viability under MAS; the immunoblot showing the O-acetylation state of the O-antigen at increasing cell growth phase; the evolution of the NMR spectra within a sealed MAS rotor; the spectral region 2.2−2.0 ppm of the spectra in Figure 4; the full NMR spectra whose anomeric region is shown in Figures 2 and S8; the NMR spectrum of the isolated LPS; tables with NMR chemical shifts of the O-antigen. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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