Rapid Analysis of *Listeria monocytogenes* Cell Wall Teichoic Acid Carbohydrates by ESI-MS/MS

Marcel R. Eugster, Martin J. Loessner*

Institute of Food, Nutrition and Health, ETH Zurich, Zurich, Switzerland

**Abstract**

We report the application of electrospray ionization (ESI) mass spectrometry for compositional characterization of wall teichoic acids (WTA), a major component of Gram-positive bacterial cell walls. Tandem mass spectrometry (ESI-MS/MS) of purified and chemically hydrolyzed monomeric WTA components provided sufficient information to identify WTA monomers and their specific carbohydrate constituents. A lithium matrix was used for ionization of uncharged WTA monomers, and successfully applied to analyze the WTA molecules of four *Listeria* strains differing in carbohydrate substitution on a conserved polyribitol-phosphate backbone structure. Carbohydrate residues such as *N*-acetylglucosamine or rhamnose linked to the WTA could directly be identified by ESI-MS/MS, circumventing the need for quantitative analysis by gas chromatography. The presence of a terminal *N*-acetylglucosamine residue tethered to the ribitol was confirmed using fluorescently labeled wheat-germ agglutinin. In conclusion, the mass spectrometry method described here will greatly facilitate compositional analysis and characterization of teichoic acids and similar macromolecules from diverse bacterial species, and represents a significant advance in the identification of serovar-specific carbohydrates and sugar molecules on bacteria.

**Introduction**

Teichoic acids are anionic carbohydrate-containing polymers present in the cell wall of many Gram-positive bacteria, and encompass both wall teichoic acids (WTAs) as well as lipoteichoic acids (LTAs). The WTAs are covalently bound to the peptidoglycan by phosphodiester bonds between *N*-acytymuramic acid and a special linkage unit, whereas LTAs are amphipathic molecules tethered to the cytoplasmic membrane via a glycolipid moiety \[1,2,3,4,5\]. In general, membrane-associated LTAs show less structural and compositional diversity than WTAs \[6,7\].

Peptidoglycan-associated WTAs are highly variable in structure, and often feature species- or even strain-specific variations. Most frequently, they are comprised of polyglycerol-phosphate (GroP) or polyribitol-phosphate (RboP) chains both of which can be decorated with a variety of different sugars and/or esterified with d-alanine \[8,9\]. WTAs composed of poly(RboP) are produced by important human pathogens such as *Staphylococcus aureus*, *Staphylococcus saprophyticus*, and *Listeria monocytogenes* \[10\]. The fact that WTA polymers are variable in structure suggests that different polymers may display distinct functions.

In *Listeria*, broad structural diversity of WTAs is brought about by variation of the glycosidic substituents attached to a polyribitol-phosphate backbone \[13,14,15,16,17\]. The species *L. monocytogenes* features WTAs composed of repeating RboP units mainly varying in carbohydrate components such as *N*-acetylglucosamine (GlcNAc), rhamnose (Rha), glucose (Glc), and galactose (Gal), depending on the *Listeria* strain and serovar. WTAs from strains belonging to serovar group 1/2 and 3 feature Rha (in serovar 1/2) and GlcNAc (in serovar groups 1/2 and 3) as substitution on the C2 and C4 positions of the ribitol molecule. In contrast, strains of serovars 4, 5, and 6 show a more complex WTA structure in which GlcNAc is incorporated as a part of the polyribitol chain, and may bear Glc and/or Gal substituents \[16,18\]. So far, d-alanine substitution has not been reported to occur in *Listeria* WTA \[15\].

In order to be able to determine the role and function of WTAs in this and other bacteria more precisely, it is fundamental to obtain compositional and structural information of WTAs of wild-type strains or mutants producing altered WTA or lacking certain cell wall glycopolymers. Until now, WTA analysis has mostly been based upon laborious chemical analysis of degradation products by gas chromatography (GC) \[13\], and structure elucidation by \[^{13}\text{C}\] and \[^{2}\text{H}\] nuclear magnetic resonance (NMR) spectroscopy \[9\]. Therefore, the aim of this study was to develop a rapid and simple method for the analysis of WTA repeating units and their carbohydrate components by application of electrospray ionization...
tation tandem mass spectrometry (ESI-MS/MS), which would be applicable for investigating a larger number of bacterial strains and/or corresponding mutants.

Results

Based on the serovar, different Listeria strains were chosen for the analysis of their WTA composition. Listeria strains EGDe, WSLC 1442, and WSLC 1485 were used as strains with GlcNAc and/or Rha substituents present on ribitol. Strain WSLC 1042 was analyzed as a member of serovar group 4, 5, and 6, where GlcNAc is an integral component of the WTA backbone chain.

Preparation of wall teichoic acids

WTAs of strains EGDe, WSLC 1485, WSLC 1442, and WSLC 1042 isolated from purified cell walls by mild extraction with a glycine/HCl buffer resulted in water soluble, crude WTA preparations. These extracts were further purified by anion exchange chromatography on a HiTrap DEAE-Sepharose Fast Flow column. The components retained by DEAE-Sepharose Fast Flow showed high absorbance at 205 nm after elution by a NaCl gradient. Fractions were assayed for total phosphate content to identify WTA containing fractions. In all cases, WTAs eluted as a single peak in accordance with the major absorbance signal at 205 nm. Similar elution profiles were obtained for all investigated Listeria strains (data not shown). Phosphate-containing fractions were pooled and desalted by dialysis. For detailed compositional analysis, purified WTA polymers were hydrolyzed with hydrogen fluoride (HF) to cleave the phosphodiesters bonds of the polyribitol subunits and obtain WTA monomers. The glycosidic bonds between ribitol and sugar substituents are not affected by HF treatment at 0°C [19].

Mass spectrometry analysis of wall teichoic acid monomers

The composition of WTA monomers was determined by ESI-MS/MS. Overall, mass spectrometry findings for all WTA samples were in agreement with the previously known components of the WTAs specific for each of the different serovars. Figure 1 and Figure 2 show the mass spectra of WTA monomers, and corresponding monomeric structures as well as fragmentation patterns as the result of electrospray ionization. Compositional data were obtained by ESI-MS/MS analysis of basic peaks of monomeric WTA samples, and a synopsis of the results obtained for each of the analytes is presented in Table 1.

In case of WSLC 1442 and WSLC 1042, samples were prepared with lithium trifluoroacetate (Li-TFA) to be able to measure a signal in the positive ion mode, whereas WTAs from strains EGDe and WSLC 1485 were measured as protonated molecules [M+H]^+*. Each WTA type analyzed in this work revealed a characteristic MS spectrum and MS/MS fingerprint for the sample molecule. Representative spectra obtained from EGDe, WSLC 1485, and WSLC 1442 WTA are shown in Figure 1. In all cases, ribitol was substituted by GlcNAc and/or Rha residues.

WTAs of strain EGDe feature both GlcNAc and Rha residues on ribitol, resulting in a total monomeric mass [M+H]^+ of m/z 502.22 (Figure 1 A). As can be seen in the corresponding ESI-MS/MS spectrum (Figure 1 B), peaks at m/z 356.15 and 204.08 confirm the presence of both Rha and GlcNAc. While m/z 356.15 is consistent with the fragment remaining upon cleavage of Rha (−146), m/z 204.08 represents the GlcNAc residue left after fragmentation between GlcNAc and ribitol. Note that m/z 204.08 may be derived from m/z 502.22 (loss of ribitol and rhamnose, −290), or m/z 356.15 (loss of ribitol, −152).

Peaks in the MS and MS/MS spectra of strain WSLC 1485 (Figure 1 D, E, and F) are nearly identical to the spectra of strain EGDe. Peak m/z 502 is absent, due to the lack of Rha in WTAs of strain WSLC 1485. Instead, the mass spectrum of WTA sample 1485 is dominated by a major peak at m/z 356.15, representing the protonated monomer [M+H]^+ of WTA 1485 (molecular weight 355.34). In addition, ions with m/z 338.14 and 378.13 could be observed: the peak at m/z 338.14 is ascribed to the loss of a single water molecule (−18) from m/z 356.15, and the peak at m/z 378.13 corresponds to the Na^+ adduct of the WTA 1485 monomer. In ESI-MS/MS, the [M+H]^+ ion at m/z 356.15 produced m/z 204.09, which can be attributed to a GlcNAc residue.

For strain WSLC 1442, the component at m/z 305.15 characterizes a Li^+ adduct (m/z +7), reflecting the monomeric WTA unit of strain WSLC 1442 (Figure 1, parts G, H and I). ESI-MS/MS analysis yielded a principal fragmentation peak at m/z 159.09 clearly corresponding to ribitol. These data confirm the presence of Rha in WTA 1442, which had been lost during fragmentation (−146). No GlcNAc could be detected in the spectra of strain WSLC 1442.

WTAs of strain WSLC 1042 have a more complex and unique structure: the GlcNAc residues are not only incorporated in the polyribitol phosphate chain, they also feature further modification by addition of Glc or Gal residues. Figure 2 A illustrates the data obtained by mass spectrometry analysis of WTA 1042. The prominent peak at m/z 686.28 corresponds to the Li^+ adduct of the WTA monomer. After fragmentation by ESI-MS/MS, the major peak at m/z 524.23 (Figure 2 B) reflects elimination of the Glc or Gal substitution from the central GlcNAc, both producing the same molecular weight. The weak signal at m/z 372.16 likely arose from the loss of the ribitol moiety (−152), as well as a Glc or Gal residue (−162), resulting in a lithiated GlcNAc ion still carrying either Glc or Gal.

Binding of wheat germ agglutinin to the bacterial cell surface

The wheat germ agglutinin (WGA) lectin requires terminal GlcNAc residues on WTAs for attachment [20]. To confirm the presence of terminal GlcNAc residues on WTAs of serovar 1/2 and 3 strains, we used fluorescent WGA. Phase contrast and corresponding fluorescence images of WGA binding to strains of serovar 1/2 and 3 are depicted in Figure 3 and summarized in Table 1. WGA showed a strong association with the entire cell wall of EGDe and WSLC 1485, whereas WSLC 1442 cells were not decorated.

Discussion

Cell wall teichoic acids represent the major surface glycopolymers of many Gram-positive bacteria. Depending on the organism, they consist of polyglycerol-phosphate or polyribitol-phosphate, decorated with d-alanine esters or mono- and disaccharides [2]. The presence of WTAs on the bacterial cell wall affects the physiology of the bacterium and the interaction with its environment. Recently, WTAs have been increasingly studied because of their roles in biofilm formation, host cell interaction and virulence in human pathogens such as the staphylococci. WTA synthesis and linkage represent possible targets for anti-infective drugs, antibiotics, and diagnostics [7,10,21]. However, most of the biological functions of WTAs are still not well understood.
Structural and compositional analysis of WTAs in bacterial wild-type strains and mutants had been a challenging task, which was based mainly upon gas chromatography and $^{13}$C and $^1$H NMR spectroscopy. Both methods are well-established and reliable, but laborious and time-consuming. Mass spectrometry had recently been employed to study LTAs [22,23]. We here report a rapid, sensitive and specific tandem mass spectrometric technique for the characterization of WTAs and their carbohydrate substituents, which can be applied to analyze minor compositional differences of various WTA polymers. ESI-MS requires gas-phase ions for analysis. In most cases, however, polymers cannot readily be converted to gas-phase species because of the strong macromolecular structure and bonds. The creation of ion fragments by a subsequent MS/MS step is useful for determining the chemical composition of the molecule. Therefore, our aim was to elucidate the structure of WTA polymers and fragmented building blocks by ESI-MS and ESI-MS/MS.

For this purpose, WTAs extracted from Listeria cell walls were first purified by anion exchange chromatography, a prerequisite for quality analysis. Monitoring total phosphate content in the fractions using commercially available reagents and phosphate tests allowed rapid screening of many samples, and represents a much more convenient approach compared to previously used, time-consuming ashing procedures [24,25]. Prior to analysis by ESI-MS and ESI-MS/MS, the WTA polymers were hydrolyzed to monomers and directly analyzed by tandem mass spectrometry in positive ion mode. Subsequent ESI-MS/MS tandem analysis permitted detection of major components based on fragment ions in the spectrum, which enabled direct identification of the individual carbohydrate moieties of WTA monomers.

As a proof of concept, four different Listeria strains were selected for detailed WTA analysis: EGDe, WSLC 1485, and WSLC 1442 differ in carbohydrate substitution on an otherwise identical ribitol backbone structure, whereas WSLC 1042 features a different WTA type with GlcNAc embedded into the polymer chain. All
WTA samples could be analyzed in positive ionization mode, and ESI spectra all yielded strong signals for the [M+H]+ or the [M+Li]+ cations. WTA samples devoid of terminal GlcNAc residues were ionized using lithium TFA as matrix component, generating Li+[26] adducts with the WTA monomers. Results of mass spectrometry-based WTA analysis were in agreement with previously published data [13,14,15,16,26,27]. Due to structural similarities of the different WTA types, the analytes produced similar but still characteristic ion spectra. For strain WSLC 1442, our data confirmed the unusual GlcNAc deficiency in WTA molecules. In general, GlcNAc could be directly identified by ESI-MS and ESI-MS/MS as a main component of serovar 1/2 WTAs, whereas traditional GC-MS based WTA analysis commonly fails to detect N-acetylated glucosamine, because of degradation of GlcNAc to glucosamine during sample preparation [15,27]. Further, the rhamnose residues present on serovar 1/2 WTA polymers were identified as monomeric rhamnosyl moieties, rather than di-rhamnosyl components as reported previously [26,28]. Moreover, the more complex WTA structure [14,16] of the serovar 4 strain WSLC 1042 could also be deduced from the ESI-MS and ESI-MS/MS data. No α-alanyl substitution could be detected in the Listeria WTA molecules by ESI-MS/MS, which confirms previous findings [15]. However, the presence of α-alanine on Listeria WTA cannot be excluded since it might be lost by cleavage of the α-alanyl ester bond during sample preparation. The products encoded by the dltABCD genes in B. subtilis [29] and S. aureus [11] are involved in the incorporation of α-alanine not only into LTA, but also WTA. Therefore, the possibility remains that the Listeria dlt gene products attach α-alanine to both LTA [30] and WTA polymers.

In addition to mass spectrometry, the presence of GlcNAc in EGDe and WSLC 1485 WTA polymers was also demonstrated using WGA, which can bind to cell walls of selected Listeria strains [20]. Recognition and binding of WGA requires non-reducing, terminal GlcNAc residues [31], such as those present on Listeria cells of serovars 1/2 and 3 WTAs, but not on the mutant strain WSLC 1442 [27]. Lack of GlcNAc in WSLC 1442 WTA as shown by ESI-MS/MS is responsible for the failure to bind the lectin. WGA also fails to bind to WSLC 1042 serovar 4 cells, where GlcNAc is not only an integral component of the poly(RboP) chain, but may be further decorated by hexose moieties such as Gal and Glc. Binding of carbohydrate-specific lectins can be exploited to determine principal presence of terminal GlcNAc on polymers such as WTAs. Similarly, highly specific cell wall binding domains of bacteriophage endolysins [20] or cell wall recognition

### Table 1. Mass spectrometry data, carbohydrate composition of the WTA repeating units, and WGA binding properties for the bacterial strains used in this study.

| Listeria strain | serovar | WTA repeating unit components | calculated molecular weight (Da) of WTA repeating units | observed mass (m/z) of WTA repeating units (basic peak) | Binding of WGA
|-----------------|---------|-----------------------------|---------------------------------|-----------------------------------|------------------|
| EGDe 1/2a       | Rbo, GlcNAc, Rha | 501.48                      | 502.22, [M+H]+                  | +                                 |
| WSLC 1485 3a    | Rbo, GlcNAc    | 355.34                      | 356.15, [M+H]+                  | +                                 |
| WSLC 1442 1/2a2 | Rbo, Rha      | 298.29                      | 305.15, [M+Li]+                 | –                                 |
| WSLC 1042 4b    | Rbo, Gal, Glc, GlcNAc | 679.62                  | 686.28, [M+Li]+                 | –                                 |

1Gal, galactose; Glc, glucose; GlcNAc, N-acetylglucosamine; Rbo, ribitol; Rha, rhamnose; in serovar 4b strain WSLC 1042, GlcNAc is incorporated in the polyribitol-phosphate chain and decorated with Gal and Glc residues.

2The calculated masses correspond to the monomeric unit of WTA, i.e., ribitol and attached carbohydrates.

3WTAs of strains EGDe and WSLC 1485 were detected as protonated molecules, whereas WTA samples of strains WSLC 1442 and WSLC 1042 were identified as lithiated molecules.

4+, binding; –, no binding; WGA: wheat germ agglutinin.

5L. monocytogenes strain WSLC 1442 is a GlcNAc-negative serovar 1/2a mutant strain [27].

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for 30 min, harvested by centrifugation (7,000 × g, 15 min, 10°C), resuspended in SM buffer (100 mM NaCl, 0 mM MgSO₄, 50 mM Tris-HCl, pH 7.5), and frozen overnight at −20°C. Thawed cells were disrupted by double passage through a One-Shot system (Constant Cell Disruption System, Northants, UK) at a pressure of 270 MPa. After removing unbroken cells and debris by centrifugation at 1,400 × g for 5 min, cell wall material was recovered by centrifugation (20,000 × g, 30 min, 4°C), washed three times with water, and resuspended in SM buffer. The resulting fraction was treated with DNase/RNase at room temperature for 3.5 h, and incubated with proteinase K for another 2 h with gentle mixing to remove proteins (each enzyme at a final concentration of 100 µg per gram wet crude cell walls). Cell walls were then extracted with boiling 4% (w/v) SDS solution for 30 min. Insoluble material was recovered by centrifugation (20,000 × g, 30 min, 20°C) and washed 5 times with sterile deionized water by repeated centrifugation and resuspension. The procedure yielded carbohydrate cell wall material devoid of proteins and lipids. The material was lyophilized and stored in water at −20°C until use.

Extraction of wall teichoic acids

To extract WTAs from cell walls, lyophilized cell walls were treated with 25 mM glycine/HCl buffer (pH 2.5) at 100°C for 10 min [14,27,36,37]. Insoluble material was removed from extracted soluble WTAs by centrifugation at 30,000 × g for 30 min and resuspended in the same buffer. The extraction was repeated twice, and pooled supernatants containing the extracted WTA material were then dialyzed (MWCO 1,000) against purified deionized water at 4°C, with three changes of water. The resulting crude WTA fraction was again lyophilized for storage.

Purification of wall teichoic acids

WTAs from the crude preparations were purified by anion exchange chromatography, as reported earlier [13,27]. Briefly, 10 mg crude WTAs dissolved in 1 ml starting buffer (10 mM Tris-HCl, pH 7.5) were applied to a HiTrap DEAE FF column (5 ml; GE Healthcare, Glattbrugg, Switzerland) previously equilibrated with the same buffer. The column was eluted with a linear gradient (approximately 20 column volumes) of 0 to 1 M NaCl in 10 mM Tris-HCl (pH 7.5) at 20°C. Fractions were collected at a flow rate of 1 ml/min, and monitored using a UV detector set to 205 nm wavelength.

Phosphate determination for teichoic acid quantification

The WTA content in various chromatography fractions was quantified as total phosphate using a combination of two methods. For oxidative decomposition, 30 µl fractions were diluted to a volume of 5 ml with distilled water, and treated with a decomposition reagent (NANOCOLOR NanOx Metal; Macherey-Nagel, Oensingen, Switzerland) according to the manufacturer’s instructions. After pretreatment, total phosphorus was determined photometrically using a phosphate test kit (Spectroquant® Phosphate Test; Merck, Zug, Switzerland). The calibration curve for quantitative measurement was established with aqueous dilutions of a 1,000 mg/l phosphate standard solution (VWR, Dietikon, Switzerland) in the range of 0.1 to 5.0 mg/l inorganic phosphate. The reaction of phosphate ions and molybdate to molybdiphosphoric acid and its subsequent reduction to phosphomolybdenum blue was monitored at 690 nm in standard 10 mm polystyrene cuvettes, after incubation for 10 min. Pure water was used as a blank. Phosphorus-containing (i.e., WTA containing) fractions were pooled, dialyzed against distilled water (three changes of water, 4°C), and lyophilized.
Acid hydrolysis of wall teichoic acids polymers

Selective cleavage of phosphodiester linkages in WTA polymers was achieved by treatment with 48% HF, as described earlier [19,30,39]. Aliquots of 1 µl l yposphorlated WTA was added to 200 µl 48% HF, and incubated in ice (0°C) for 16 h. Following removal of HF by evaporation over NaOH pellets under vacuum in a glass-free system, WTA monomers were stored in pure water at –20°C, or lyophilized for further analysis.

Mass spectrometry analysis

ESI-MS/MS analysis of WTA monomers was performed at the Functional Genomics Center Zurich (FGCZ) of ETH Zurich and the University of Zurich (Switzerland), using a Q-TOF Ultima API mass spectrometer (Micromass, UK) equipped with an electrospray ion source. Samples were measured in the positive ion mode.

For analysis of WTAs from strains WSLC 1442 and WSLC 1485, lyophilized WTA monomer samples were dissolved in a small volume of acetonitrile containing 3 mM lithium trifluoroacetate (Li-TFA), added from a 5 mM stock solution in H2O. No additive was used for the measurement of WTAs of strains EGDe and WSLC 1485. The samples were dissolved in 0.2% formic acid in 50% acetonitrile prior to measurement by ESI-MS and ESI-MS/MS. For MS/MS, argon was used as the collision gas for fragmentation of parent ions. The resulting fragment ions were separated by a time-of-flight (TOF) mass analyzer.

References

1. Araki Y, Ito E (1989) Linkage units in cell walls of Gram-positive bacteria. Crit Rev Microbiol 14: 131–155.
2. Neuhaus FC, Baddiley J (2003) A continuum of anionic charge: structures and functions of α-aminolipitol teichoic acids in Gram-positive bacteria. Microbiol Mol Biol Rev 67: 686–723.
3. Armstrong JJ, Baddiley J, Buchanen JG, Garsc B (1958) Nucleotides and the structure of the wall teichoic acids of gram-positive bacteria. Nature 181: 1692–1693.
4. Baddiley J (1972) Teichoic acids in cell walls and membranes of bacteria. Essays Biochem 8: 35–77.
5. Fischer W (1994) Lipoteichoic acids and lipoglycans. In: Ghuysen J-M, Hakenbeck R, eds. Bacterial cell wall Elsevier Science Publishing B.V., Amsterdam, The Netherlands. pp 199-216.
6. Fischer W (1988) Physiology of lipoteichoic acids in bacteria. Adv Microb Physiol 29: 233–302.
7. Wiedenmaier C, Pechel A (2008) Teichoic acids and related cell-wall glycolipomers in Gram-positive physiology and host interactions. Nat Rev Microbiol 6: 276–287.
8. Navarre VW, Schrenwin O (1999) Surface proteins of Gram-positive bacteria and mechanisms of their targeting to the cell wall envelope. Microbiol Mol Biol Rev 63: 174–229.
9. Naumova IB, Shashkov AS, Tuf’skaya EM, Shrestiakva GM, Kozlova YI, et al. (2001) Cell wall teichoic acids: structural diversity, species specificity in the genus Lactobacillus, and chemotaxonomic perspective. FEMS Microbiol Rev 25: 269–284.
10. Xia G, Pechel A (2008) Toward the Pathway of S. aureus WGA Biosynthesis. Chem Biol 15: 95–96.
11. Pechel A, Otto M, Jack RW, Kallberger H, Jung G, et al. (1999) Inactivation of the dfr operon in Staphylococcus aureus confers sensitivity to defensins, protegrins, and other antimicrobial peptides. J Biol Chem 274: 8405–8410.
12. Wiedenmaier C, Kokai-Kun JF, Kristian SA, Chantuya T, Kallberger H, et al. (2004) Role of teichoic acids in Staphylococcus aureus nasal colonization, a major risk factor in nosocomial infections. Nat Med 10: 243–245.
13. Felder F, Seger J, Schreiterbunner A, Seeliger HPR (1984) The Biochemistry of Murein and Cell-Wall-Tecicho-Acids in the Genus Listeria. Systematic and Applied Microbiology 3: 360–376.
14. Fuji H, Kaminagka K, Nagaoka M, Uchikawa K, Sekikawa I, et al. (1983) Structural study on teichoic acids of Listeria monocytogenes types 4a and 4d. J Biochem (Tokyo) 93: 1401–1409.
15. Uchikawa K, Sekikawa I, Azuma I (1986) Structural studies on teichoic acids in cell walls of several serotypes of Listeria monocytogenes. J Biochem (Tokyo) 99: 315–327.
16. Ullmann WW, Cameron JA (1969) Immunochromaty of the cell walls of Listeria monocytogenes. J Bacteriol 96: 406–493.
17. Binding assay and fluorescence microscopy

A fluorescent WGA-Alexa Fluor594 conjugate (Invitrogen, Basel, Switzerland) was used to detect terminal GlcNAc residues on Listeria WTA [20,31]. For this purpose, Listeria cells were harvested from late log phase cultures by centrifugation at 7,000× g for 60 s and resuspended in 1/10 volume PBST. One-hundred µl samples were mixed with 50 µl of WGA solution (0.1 mg/ml) and incubated for 5 min at room temperature. Bacterial cells with bound lectin were recovered by centrifugation at 16,000× g for 1 min, and washed twice in 500 µl of PBST. The pellet was resuspended in 50 µl of PBST buffer and observed using confocal laser scanning microscopy (Leica TCS SPE, Leica, Heerbrugg, Switzerland).

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Author Contributions

Conceived and designed the experiments: MRE MJL. Performed the experiments: MRE. Analyzed the data: MRE MJL. Contributed reagents/materials/analysis tools: MRE MJL. Wrote the paper: MRE MJL.
35. Valyasevi R, Sandine WE, Geller BL (1990) The bacteriophage K receptor of \textit{Lactococcus lactis} subsp. cremoris KH is the rhamnose of the extracellular wall polysaccharide. Appl Environ Microbiol 56: 1882–1889.

36. Kaya S, Araki Y, Ito E (1985) Structural studies on the linkage unit between poly(galactosylglycerol phosphate) and peptidoglycan in cell walls of \textit{Bacillus coagulans}. Eur J Biochem 147: 41–46.

37. Kojima N, Araki Y, Ito E (1983) Structure of linkage region between ribitol teichoic acid and peptidoglycan in cell walls of \textit{Staphylococcus aureus} H. J Biol Chem 258: 9043–9045.

38. Fiedler F, Schaffler MJ, Stackebrandt E (1981) Biochemical and Nucleic Acid Hybridisation Studies on \textit{Brevibacterium flavum} and Related Strains. Arch Microbiol 129: 85–93.

39. Lipkin DI, Phillips BE, Ahrell JW (1969) Action of hydrogen fluoride on nucleotides and other esters of phosphorus(V) acids. J Org Chem 34(6): 1539–1547.