RESEARCH LETTER

Listeria monocytogenes tyrosine phosphatases affect wall teichoic acid composition and phage resistance

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Abstract

Tyrosine phosphatase (PTP)-like proteins exist in many bacteria and are segregated into two major groups: low molecular weight and conventional. The latter group also has activity as phosphoinositide phosphatases. These two kinds of PTP are suggested to be involved in many aspects of bacterial physiology including stress response, DNA binding proteins, virulence, and capsule/cell wall production. By annotation, *Listeria monocytogenes* possesses two potential low molecular weight and two conventional PTPs. Using *L. monocytogenes* wild-type (WT) strain 10403S, we have created an in-frame deletion mutant lacking all four PTPs, as well as four additional complemented strains harboring each of the PTPs. No major physiological differences were observed between the WT and the mutant lacking all four PTPs. However, the deletion mutant strain was resistant to *Listeria* phages A511 and P35 and sensitive to other *Listeria* phages. This was attributed to reduced attachment to the cell wall. The mutant lacking all PTPs was found to lack *N*-acetylglucosamine in its wall teichoic acid. Phage sensitivity and attachment was rescued in a complemented strain harboring a low molecular weight PTP (LMRG1707).

Introduction

In recent years, accumulated data suggest that bacteria possess tyrosine kinases, phosphatases, and tyrosine phosphorylated proteins (Grangeasse *et al.*, 2007). However, the role of such phosphorylation was elucidated only in a few species (Grangeasse *et al.*, 2007). In Gram-negative bacteria, many tyrosine kinases and phosphatases were found (Bechet *et al.*, 2009). In *Escherichia coli*, processes associated with cell wall modifications were suggested (Grangeasse *et al.*, 2003; Peleg *et al.*, 2005; Bechet *et al.*, 2009). Phospho-proteome analysis of *E. coli* has revealed additional proteins phosphorylated on tyrosine, related to different cellular aspects including carbon metabolism and the glycolytic pathway (Macek *et al.*, 2008). Additionally, other Gram-negative bacteria (such as *Yersinia* and *Salmonella*) were shown to have tyrosine phosphatases that are secreted into their host cells via a type III secretion system (YopH and SptP) (Murli *et al.*, 2001; Cozzone, 2005; Yuan *et al.*, 2005). These phosphatases are

responsible for the manipulation of the host response to the benefit of the pathogen. In Gram-positive bacteria, tyrosine phosphorylation machinery was documented in both pathogenic bacteria (e.g. *Streptococcus pneumoniae* and *Staphylococcus aureus*) (Grangeasse *et al.*, 2007; Bechet *et al.*, 2009) and nonpathogenic bacteria (e.g. *Bacillus subtilis* and *Lactococcus lactis*) (Grangeasse *et al.*, 2007; Bechet *et al.*, 2009).

Interestingly, in *Mycobacterium tuberculosis*, it was thought that the two secreted protein tyrosine phosphatases (PTP), PtpA and PtpB, do not have a bacterial kinase counterpart and are thus responsible mostly for host manipulation (Chao *et al.*, 2010). However, a recent finding suggests that PtpA is phosphorylated on tyrosine by a newly identified nonconservative tyrosine kinase, PtkA (Bach *et al.*, 2009; Chao *et al.*, 2010).

Listeria monocytogenes is a ubiquitous facultative intracellular Gram-positive bacterium that causes invasive devastating disease mainly in older people, pregnant women (leading to abortion and fetus loss), newborns, and

immunocompromised hosts (Siegman-Igra *et al.*, 2002; Guevara *et al.*, 2009). Interestingly, *L. monocytogenes* has four PTPs without known adjacent kinase genes. These phosphatases belong to two major types – two low molecular weight PTPs and two conventional PTPs (Kastner *et al.*, 2011). Recently, it was suggested that the two conventional PTPs belong to a group of enzymes that includes the *M. tuberculosis* PtpB (Beresford *et al.*, 2010; Kastner *et al.*, 2011). This group of phosphatases is active on phosphoinositides as well as on tyrosine phosphates (Koul *et al.*, 2000; Beresford *et al.*, 2010). Lower phosphorylated serine/threonine activity was noted as well (Beresford *et al.*, 2010). In *Listeria*, it was shown that a mutant of LO28 strain deficient in one PTP (*lipA*) had lower virulence and lower bacterial counts in target organs (Kastner *et al.*, 2011). Additionally, it was suggested that such PTPs might serve as a target for new antibiotics, mainly for the intracellular pathogen *M. tuberculosis* (Grundner *et al.*, 2007; Beresford *et al.*, 2009; Zhou *et al.*, 2010). Thus, understanding the role of PTPs in *L. monocytogenes* should also elucidate its role in other pathogenic and intracellular bacteria.

Materials and methods

Bacterial strains

The *L. monocytogenes* strains used (see Table 1) were a wild-type strain (WT), 10403S, or a strain containing an in-frame deletion of each of the PTP (DP-L5359). These deletions were generated by sequential deletion of each of the phosphatases using splice-overlap extension (SOE)-PCR and allelic exchange, as described elsewhere (Camilli *et al.*, 1993) using the primers in the Supporting Information, Table S1. Complemented strains harboring only one of each of the phosphatases were generated using the pPL2 integrational vector (Lauer *et al.*, 2002) and the primers in Table S1 to synthesize the PTP genes. *Listeria monocytogenes* DP-L861, also known as Mack (Hodgson, 2000), was used for phage propagation.

Bioinformatics

Nucleotide and amino acid sequence analyses and interpretation were carried out using Vector NTI Advance (Invitrogen, Basel, Switzerland). Pairwise sequence align-ments were made using the BLASTn, BLASTp, and tBLAST programs available at the NBCI website. The multiple alignment was made using CLUSTALW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The program BOXSHADE 3.21 (http://www.ch.embnet.org/software/BOX_form.html) was used to generate white letters on black boxes for residues that are identical and white letters on gray boxes for residues that are similar in sequences.

Bacterial intracellular growth curves

Bacterial intracellular growth curves were determined as described previously (Portnoy *et al.*, 1988). Briefly, 2×10^6 bone marrow–derived macrophages (BMDM) were infected with 4×10^5 CFU of *L. monocytogenes* from an overnight culture. Thirty minutes after the addition of bacteria, macrophage monolayers were washed with PBS. One hour postinfection, gentamicin was added to $50 \ \mu g \ mL^{-1}$ to kill the extracellular bacteria. At different time points postinfection, three coverslips were taken and washed with water to lyse host cells. Bacteria recovered from each coverslip were plated on brain heart infusion (BHI) plates, and the number of CFU was determined.

Phage growth

A511 was prepared according to Loessner & Scherer (1995). A118 and U153 were prepared as described for A118 by Loessner *et al.* (2000), except that the host strain was DP-L861. P35 (Hodgson, 2000) was prepared as a plate stock, using Luria–Bertani (LB) plates supplemented with 5 mM $CaCl_2$. The stock was sterilized by filtration through pores of 0.4 μm diameter.

Phage adsorption assays

Standing cultures of bacteria were grown in BHI overnight at 30 °C. The cell concentrations were $> 10^8 \ mL^{-1}$; 40 μL of cells was mixed with 1 μL of A511 ($4 \times 10^7 \ mL^{-1}$) and 1 μL of 0.5 M $CaCl_2$. The mixture was incubated for 15 min at 30 °C, and the bacteria were removed by centrifugation. We assayed phage remaining in the supernatant on BHI plates, using DP-L861 as indicator.

Phage plaquing efficiency was determined by titrating 100-fold dilutions of various *Listeria* phages (A511, P35,

Table 1. *Listeria* strains used in this study

Strain	10403S	DP-L5359	DP-L5412	DP-L5413	DP-L5414	DP-L5415
Description	WT strain	10403S, ΔLMRG2037, ΔLMRG0947, ΔLMRG1082, ΔLMRG1707	DP-L5359 tRNA^{arg}::LMRG0947	DP-L5359 tRNA^{arg}::LMRG1082	DP-L5359 tRNA^{arg}::LMRG2037	DP-L5359 tRNA^{arg}::LMRG1707

U153, and A118) with the strains described in this study. The numbers of plaques were compared with the numbers obtained with the WT strains 10403S and DP-L861. Plaques were enumerated after incubation at 30 °C for 24 and 72 h.

Sensitivity of *L. monocytogenes* to bacteriophage lysin was determined as was previously described (Loessner *et al.*, 1996). Briefly, stationary *L. monocytogenes* strains were washed twice with PBS and resuspended in 50 mM Na_2HPO_3 at $A_{600\,nm}$ of 1. Then, strains were exposed to A511 Ply (bacteriophage lysin) (Loessner *et al.*, 1996) at a final concentration of 1 U mL^{-1} and were followed for change at optical density (OD) $A_{600\,nm}$ absorbance for 90 min.

Preparation of cell walls

Cell walls were purified as previously described (Fiedler *et al.*, 1984; Valyasevi *et al.*, 1990; Eugster & Loessner, 2011). Bacterial strains were grown in BHI broth to an $A_{600\,nm}$ of 0.8 and inactivated by heating to 100 °C for 20 min. Cells were harvested by centrifugation (7000 ***g***, 10 min, 4 °C), resuspended in SM buffer (100 mM NaCl, 10 mM $MgSO_4$, 10 mM Tris–HCl, pH 7.5), and disrupted by passing through a French Press at 270 MPa. Unbroken cells were sedimented by centrifugation at 1400 ***g*** for 5 min, and crude cell walls were washed twice with water and resuspended in SM buffer. The crude cell wall fraction was treated enzymatically with DNase and RNase at room temperature for 3 h and subsequently incubated with proteinase K for another 2 h (enzyme concentrations: 100 µg g^{-1} wet crude cell walls). Further, cell walls were boiled in 4% sodium dodecyl sulfate (SDS) for 30 min and recovered by centrifugation (30 000 ***g***, 30 min, 20 °C), and the pellet was washed five times with water to remove residual SDS. The resulting preparation was lyophilized and used for the determination of total cell wall phosphate content.

Determination of cell wall phosphate content

To measure total cell wall phosphate content, samples were assayed as published earlier (Eugster & Loessner, 2011). A 10-µL sample of a 10 mg mL^{-1} purified cell wall suspension was first digested oxidatively using a NANOCOLOR® NanOx Metal (Macherey-Nagel) according to the manufacturer's protocol. Then, total phosphorus was determined photometrically by the use of a phosphate test kit (Spectroquant® Phosphate Test; Merck) as described by the manufacturer. To assure the accuracy and reliability of the results, a calibration curve was obtained with aqueous dilutions of a 1000 mg L^{-1} phosphate standard solution (VWR). All samples were decomposed and measured in triplicate.

Detection of *N*-acetylglucosamine in wall teichoic acids

Wheat germ agglutinin (WGA)-Alexa Fluor 594® conjugate (Invitrogen) was used for the detection of *N*-acetylglucosamine (GlcNAc) in wall teichoic acids (WTA) of *Listeria* cells. This lectin recognizes terminal GlcNAc substituents in cell wall polymers, such as WTA on the surface of *L. monocytogenes* (Wright, 1984; Loessner *et al.*, 2002; Eugster & Loessner, 2011). Binding assays with labeled WGA were performed as described elsewhere (Loessner *et al.*, 2002; Eugster & Loessner, 2011). Bacterial cells were harvested in late log phase by centrifugation and resuspended in 1/10th volume of PBST buffer (120 mM NaCl, 50 mM phosphate, and 0.1% Tween 20, pH 8.0); 100 µL cells and 50 µL of Alexa Fluor 594® WGA solution (0.1 mg mL^{-1}) were mixed and incubated for 10 min at 25 °C. Cells were removed from labeling solution by centrifugation (12 000 ***g***, 1 min) and washed twice in PBST buffer. After washing, the cells were examined by fluorescence microscopy (Leica TCS SPE; Leica, Heerbrugg, Switzerland). Additionally, the presence of GlcNAc was tested using GFP-labeled cell wall-binding domain (CBD) of bacteriophage endolysin PlyP35 (HGFP-CBDP35), which specifically recognizes GlcNAc residues in *Listeria* WTA (Eugster *et al.*, 2011). Binding assays with HGFP-CBDP35 were performed as described earlier (Loessner *et al.*, 2002; Schmelcher *et al.*, 2010; Eugster *et al.*, 2011).

Statistical analysis

All experiments were repeated at least twice to confirm reproducibility. Categorical data were compared using the chi-square test or the Fisher's exact test when appropriate. Continuous variables were compared using the Mann–Whitney *U*-test or Student's *t*-test if number of repetitions was < 5.

Results

Bioinformatics analysis of tyrosine phosphatases in *L. monocytogenes*

Listeria monocytogenes harbors four putative tyrosine phosphatases (LPTP): two of them belong to the low molecular weight phospho-tyrosine phosphatases and are annotated as LMRG2037 (LptpA1) and LMRG1707 (LptpA2) (Fig. 1). An additional two belong to the conventional weight phospho-tyrosine phosphatases and are annotated as LMRG0947 (LptpB1; lipA, LMO1800 in *L. monocytogenes* strain EGDe) and LMRG1082 (LptpB2,

LMO1935 in *L. monocytogenes* strain EGDe) and described in detail recently (Beresford *et al.*, 2010; Kastner *et al.*, 2011).

All four tyrosine phosphatases are highly conserved within all strains of *Listeria* species that were fully sequenced to date (Table 2). All four PTP-coding genes were found in all sequenced strains of *Listeria* except for LptpA2, which was missing in the published fully sequenced *L. monocytogenes* LO28 isolate (serotype 1/2c). In the only sequenced *Listeria grayi* isolate, both conventional PTPs are missing; however, the genome of this isolate contains two other conventional PTPs that have no homologs in other *Listeria* strains. An operon that is homologous to the operon of LptpA2 was found in *B. subtilis* (Musumeci *et al.*, 2005) and in other Gram-positive bacteria such as *S. aureus* (Musumeci *et al.*, 2005). Additionally, LptpA1 has 51% amino acid similarity and 31% aa identity to PtpA of *M. tuberculosis* (Fig. 1a) and is suggested to be a secreted PTP (Bach *et al.*, 2008).

Creation of tyrosine phosphatases deletion mutants

To study the specific role of each phosphatase and to prevent a possible cross-reactivity and specificity as is suggested by the sequence homology, we have created a *L. monocytogenes* mutant lacking all PTPs (DP-L5359). This was achieved by sequential deletions of all four phosphatases in the WT strain 10403S. We also have created single gene complemented strains, using the pPL2 integration vector as previously described (Lauer *et al.*,

2002). All strains used in this study are presented in Table 1.

Tyrosine phosphatases in a variety of conditions in *L. monocytogenes*

We looked for differences in *L. monocytogenes* physiology between the WT and the PTPs knock-out strain. We did not observe a growth defect in BHI or LB at either 37 or 30 °C (data not shown except for BHI 30 °C, Fig. 2a). In a previous report, it was suggested that *B. subtilis* lacking a low molecular PTP is more sensitive to ethanol stress (Musumeci *et al.*, 2005). However, the DP-L5359 grew without significant difference compared with WT in the presence of 5% ethanol (Fig. 2b). Additionally, DP-L5359 was able to resist oxidative stress (100 mM H_2O_2) more efficiently than the WT (Fig. 2c). To assess whether cell wall integrity is impaired, we looked at differences in susceptibility to mutanolysin of the different *L. monocytogenes* strains. DP-L5359 was more resistant to mutanolysin, as was noticed by reduced clearance of turbidity after exposure to 100 mM mutanolysin (Fig. 2d). No differences were observed after exposure of strains to lysozyme (Fig. S1). DP-L5359 also had a small swarming motility defect, as was shown by its reduced ability to spread on BHI soft agar (10% reduction in motility, $P = 0.045$). Growth curves of both WT and PTPs knock-out strain (DP-L5359) in BMDM revealed similar growth curves without any major growth defect for the mutant (Fig. 2e). No differences in growth curves were observed in IFN-γ-activated BMDM (Fig. S2). Similarly, no difference in
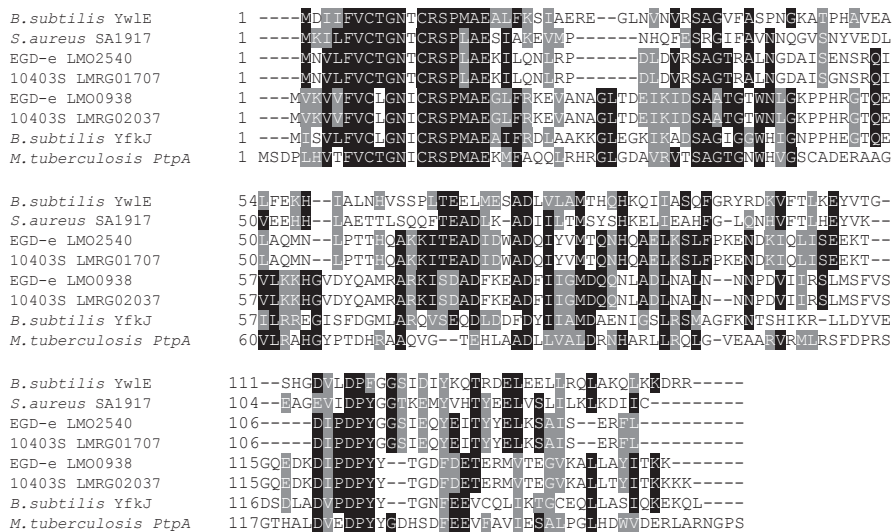

Fig. 1. The amino acid alignments of two *Listeria monocytogenes* WT (10403S and EGD-e) PTP. Comparison of the two low molecular weight PTP of both *L. monocytogenes* with those of *Bacillus subtilis* YwlE (gi|2636218) and YfkJ (gi|2633112); *Mycobacterium tuberculosis* PtpA (gi|1261936|); and *Staphylococcus aureus* low molecular weight PTP (gi|14247888|).

Table 2. Genomes of *Listeria* species harboring putative tyrosine phosphatases by annotation

	Names of sequenced genomes in the NCBI database	LptpA1 LMRG 2037 (LMO 0938)	LptpA2 LMRG 1707 (LMO 2540)	LptpB1 LMRG 0947 (LMO 1800)	LptpB2 LMRG 1082 (LMO 1935)
L. monocytogenes serotype 1/2a (9 genomes)	08-5578 08-5923 10403S EGD-e F6900 N3-165 J0161 J2818 F6854	9	9	9	9
L. monocytogenes serotype 1/2b (4 genomes)	J1-194 J1-175 J2-064 R2-503	4	4	4	4
L. monocytogenes serotype 1/2c (2 genomes)	R2-561 LO28	2	1	2	2
L. monocytogenes serotype 3a (1 genome)	Finland 1988	1	1	1	1
L. monocytogenes serotype 4a (2 genome)	HCC23 M7	2	2	2	2
L. monocytogenes serotype 4b (5 genomes)	F2365 H7858 HPB2262 J1816 N1-017 scottA Clip80459	7	7	7	7
L. monocytogenes serotype 4c (1 genome)	J2-071 L99	2	2	2	2
Listeria innocua (1 genome)	Clip11262	1	1	1	1
Listeria seeligeri (1 genome)	SLCC3954	1	1	1	1
Listeria welshimeri (1 genome)	SLCC5334	1	1	1	1
Listeria grayi (1 genome)	DSM 20601	1	1	0	0

Distribution of putative tyrosine phosphatases according to the annotation of 31 sequenced *Listeria* species available at the NCBI database. Similarity was identified using BLASTp suite (http://blast.ncbi.nlm.nih.gov/Blast.cgi). For each putative tyrosine phosphatase, the *L. monocytogenes* 10403S genome numbering was used (EGDe LMO number was provided in parenthesis) and a number of genomes that harbor the putative protein are noted. The full list of strains, serotypes and GenBank access numbers can be found in Table S2.

growth curve was also observed in epithelial cell lines (CaCo2 and HepG2, data not shown). Additionally, DP-L5359 had no virulence defect compared with the WT 10403S in the mouse model of infection (Fig. S3).

Phage susceptibility

Bacteriophages have a life cycle that involves many bacterial physiological aspects: phages adsorb to the bacterial cell wall, then penetrate into the cell, replicate using bacterial machinery for both nucleic acids and proteins, mature and reassemble new phages, break the cell wall using lysozyme-like enzymes, and release progeny virions. Therefore, phages are useful tools for evaluating possible changes affected by many processes. We tested our WT

(10403S strain), deletion mutant, and complemented strains for susceptibility to *Listeria* phages. No differences were found using phages U153 and A118. However, A511 showed an extremely reduced plaquing efficiency on the PTPs deletion mutant DP-L5359, with phenotype restoration in the strain complemented with LMRG1707 LptpA2 (Fig. 3a). A similar observation was noted with phage P35 (data not shown). Thus, the lack of PTPs blocks the phage infection cycle, and LptpA2 restores phage growth. Both WT and knock-out strains lyse at the same rate with exposure to the purified A511 lysin (Fig. 3b), suggesting that release of the phages is not affected. To see specifically whether phage attachment is crucial for these differences, we have used a phage adsorption assay. Exposing phages to 10403S resulted in almost complete elimination of
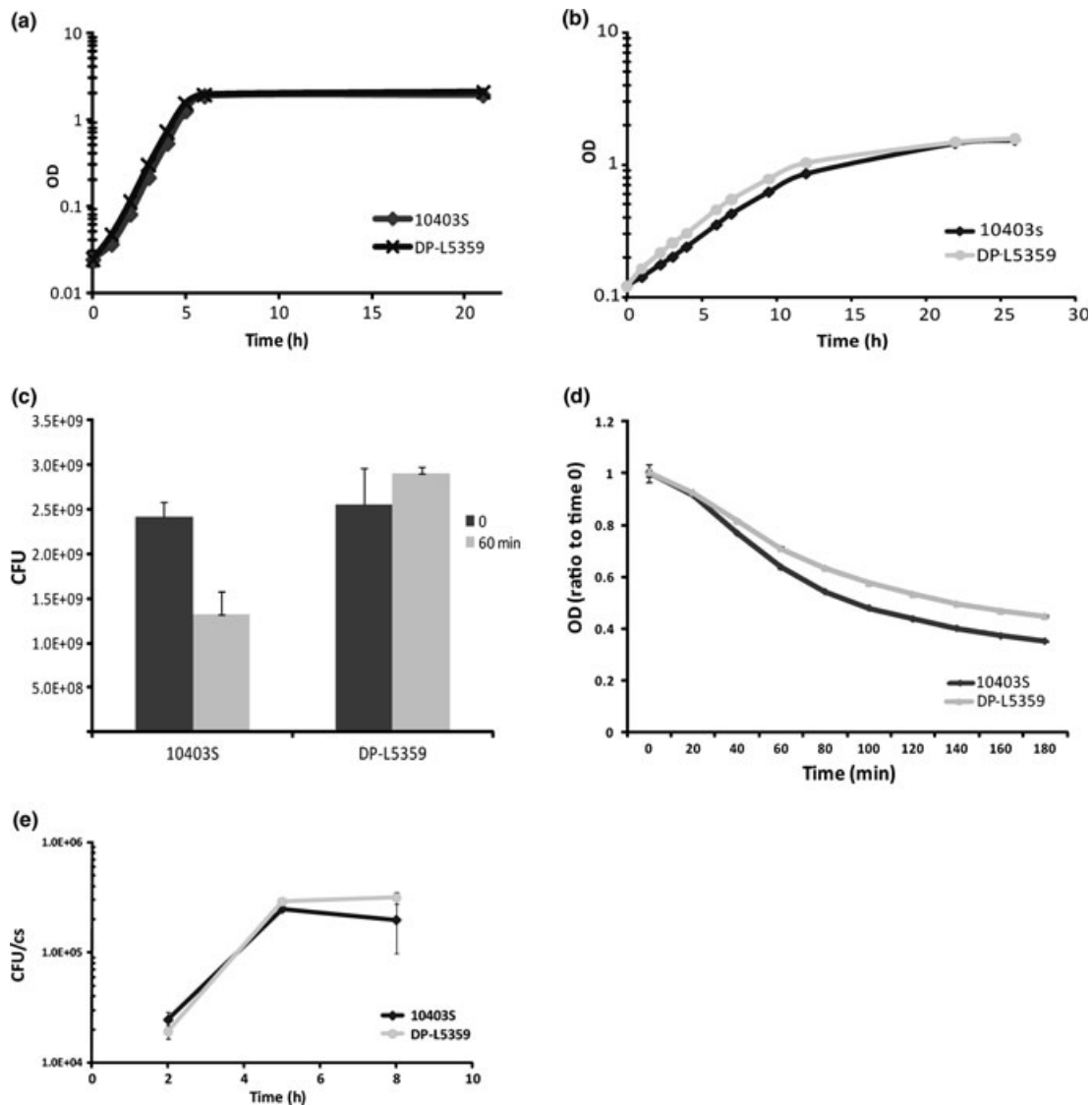
Fig. 2. Effect of tyrosine phosphorylation on *Listeria* physiology – (a) *L. monocytogenes* 10403S and DP-L5359 were grown in BHI broth 30 °C, in orbital shaker, until reaching stationary phase. The experiment was carried out in triplicate, and error bars represent SD of three experiments. (b) *L. monocytogenes* 10403S and DP-L5359 were grown in BHI broth 30 °C supplemented with 5% ethanol. DP-L5359 grew without significant difference from WT in the presence of 5% ethanol. (c) Approximately 2×10^9 CFU of both WT (10403S and PTPs KO mutant DP-L5359) was exposed to 100 mM H_2O_2 for 60 min. After 60 min, bacteria in solution were enumerated. Experiments were repeated three times, and error bars represent the SD of these triplicates. Almost 45% reduction in the viability of 10403S was noticed compared with no in DP-L5359 ($P = 0.01$). (d) 10^9 CFU late-exponential-phase *L. monocytogenes* were exposed to 100 U mL^{-1} mutanolysin over time. A small advantage was noticed for the PTPs deletion mutant (DP-L5359) over the WT (10403S) in all time points after exposure to mutanolysin ($P < 0.01$). Neither strain showed any change in absorbance up to 220 min when exposed to buffer alone (50 mM NaHPO$_3$ pH 6.8). A$_{600 nm}$ OD was measured at each time point, and SD represents a SD of three experiments. (e) Intracellular growth curves of WT *L. monocytogenes* (black) and the DP-L5359 deletion mutant (gray) in BMDM. Error bars represent one standard deviation.

phage from solution, while only very low numbers of phage were eliminated by exposing phage to DP-L5359 (Fig. 3c). This suggested to us that some differences in cell wall might be responsible for this phenotype. Interestingly, attachment was almost completely restored by one complemented strain (DP-L5415; complementation of the LMRG1707 LptpA2) and less so (~ 25%) by another complemented strain (DP-L4212; complementing with the LMRG0947 LptpB1/lipA). No complementation of attachment was observed in the other complemented strains. Thus, LptpA2 is responsible for the restoration of cell wall attachment by A511.
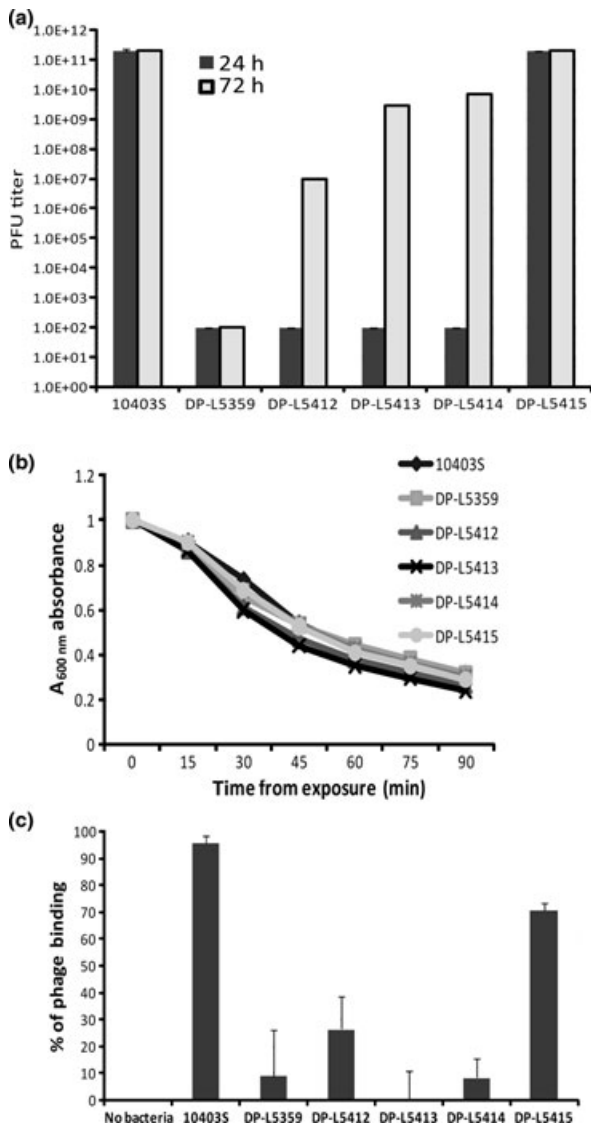
Fig. 3. The susceptibility of *Listeria monocytogenes* 10403S, PTPs deletion mutant DP-L5359, and complemented strains to *Listeria* phage A511. (a) Titer of PFU observed 24 h (dark columns) and 72 h (light gray columns) after exposing different *L. monocytogenes* strains to the same number of A511 (1.2×10^{11} PFU mL^{-1}). After 24 h only, complementation with LptpA2 (DP-L5415) enabled to restore WT phenotype. After 72 h of exposure to phage A511 of different *L. monocytogenes*, complementation with LptpB2 and LptpA1 partially restored the susceptibility. (b) Lysis of *L. monocytogenes* by purified A511 lysin is not influenced by the PTPs. Stationary *L. monocytogenes* strains were washed twice with PBS and resuspended in 50 mM Na$_2$HPO$_3$ at A$_{600\,nm}$ of 1. Then, strains were exposed to A511 ply (Loessner *et al.*, 1996) for different time periods. Similar lysis was observed in WT, DP-L5359, and four complemented strains ($P > 0.1$ comparing different strains to WT). (c) Phage adsorption can be only partially restored by only 2 PTPs. LptpA2 (DP-L5415) restores ~ 70% of WT absorption ($P = 0.001$) and LptpB1 (DP-L5412) can minimally restore absorption (20% $P = 0.14$). The two other complemented strains did not restore adsorption at all compared with DP-L5359 ($P = 1$). Error bars represent standard deviation of three repetitions. The percent of phage absorbed was correlated with the number of phages in the solution when phages were incubated without bacteria for the same time.

Identifying cell wall changes

Taken together, the phage experiments and the changes after exposure of *L. monocytogenes* to mutanolysin suggested that changes in cell wall glycopeptide might be involved. First, we have looked for changes in the teichoic acid contents of the cell wall. Purified cell walls of 10403S and deletion mutant DP-L5359 were analyzed for total phosphorus to show the presence of teichoic acids in the cell walls. Both strains provided similar values indicating similar WTA content (Fig. S4). Thereafter, we looked for changes in cell wall glycosylation. Binding of fluorescently labeled WGA, a lectin that specifically binds terminal GlcNAc residues in cell wall polymers (Lotan *et al.*, 1975),
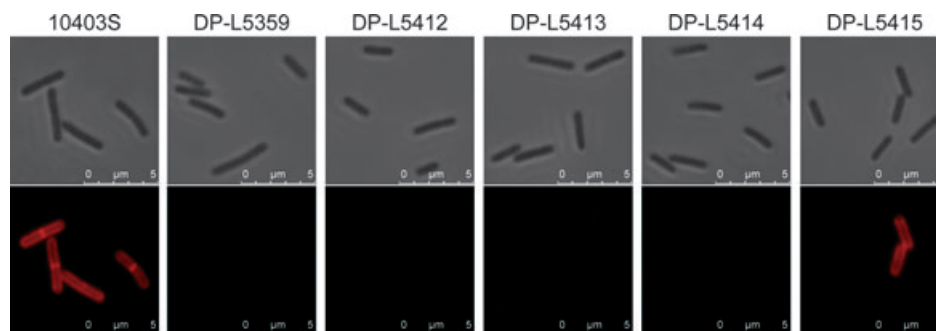

Fig. 4. Phase-contrast and corresponding fluorescence microscopy images for the detection of *N*-acetylglucosamine (GlcNAc) on WTA of *Listeria* strains 10403S, DP-L5359, and complemented strains DP-L5412, DP-L5413, DP-L5414 and DP-L5415 using Alexa Fluor 594-labeled WGA. WGA recognizes GlcNAc substitutes on WTA of *Listeria monocytogenes*. Partial restoration of 10403S is observed with complementation with LptpA2 (DP-L5415).
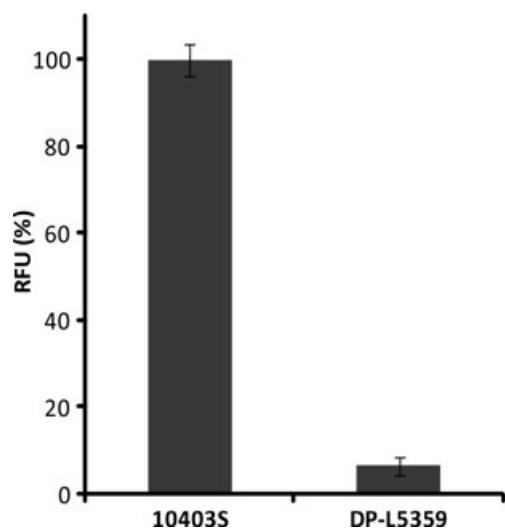
Fig. 5. Quantitative assessment of terminal GlcNAc residues in WTA by binding of GFP-tagged CBDP35 (HGFP-CBDP35). Purified cell walls of *Listeria monocytogenes* strains 10403S and DP-L5359 were incubated with HGFP-CBDP35 and analyzed for binding by quantitative fluorescence analysis. Binding is measured in relative fluorescence units (RFU), and the amount of HGFP-CBDP35 bound to cell walls of strain 10403S is defined as 100%. Measurements were taken in triplicate. Using this approach, the DP-L5359 was shown to have GlcNAc-deficient WTA.

was performed to test for the presence of GlcNAc in WTA. Alexa Fluor 594® WGA was able to stain WT strain 10403S and DP-L5415, but this lectin failed to bind to strains DP-L5359, DP-L5412, DP-L5413, and DP-L5414, pointing to a lack of GlcNAc residues in WTA (Fig. 4), which is restored in the DP-L5415 complemented with LMRG1707. The same results were obtained when binding assays were performed with GlcNAc-specific fluorescent P35 phage endolysin cell wall–binding domain HGFP-CBDP35 (Fig. 5).

Discussion

In this work, we have found that PTPs have an effect upon the composition of the *Listeria* cell wall. This is similar to many other bacteria including some pathogens (Grangeasse *et al.*, 2007; Lacour *et al.*, 2008; Bechet *et al.*, 2009). In Gram-negative bacteria tyrosine kinases and phosphorylation were suggested to be involved in the production of emulsan in the nonpathogen *Acinetobacter lwoffi* (Nakar & Gutnick, 2003) and capsular polysaccharide production in *E. coli* and a few other bacteria (Obadia *et al.*, 2007). In Gram-positive bacteria, a machinery that includes tyrosine kinase and phosphatase was sug-

gested to be involved in the synthesis and export of extracellular polysaccharides, such as *S. aureus* (Soulat *et al.*, 2002; Olivares-Illana *et al.*, 2008) and *S. pneumoniae* (Morona *et al.*, 2002). Similarly, protein tyrosine phosphorylation in *L. monocytogenes* is associated with changes in teichoic acid. However, no homologous machinery of the related Gram-positive *S. pneumoniae* or *S. aureus* can be found in *L. monocytogenes*.

The change in teichoic acids of our four PTPs deletion mutant was the lack of *N*-acetyl glucosamine (GlcNAc) in the WTA. This was demonstrated by the changes in susceptibility to *Listeria* phages and could almost completely be restored by functional LptpA2 and partially restored by LptpB1/lipA. The fact that phage A511 and the Ply of phage P35 bind GlcNAc in the WTA (Wendlinger *et al.*, 1996; Eugster *et al.*, 2011) confirms our observation. Because phage A118 adsorption is dependent on rhamnose decoration of WTA (Wendlinger *et al.*, 1996), we did not observe any changes between the A118 binding comparing the WT and the DP-L5359 strain. The lack of GlcNAc in cell WTA was further confirmed by the lack of labeling with florescent WGA or HGFP-CBDP35.

Protein tyrosine phosphatases in *Listeria* (e.g. the conventional PTPs LptpB1/LipA and LptpB2) were shown before to have dual function as tyrosine phosphatases and phosphoinositide phosphatases (Beresford *et al.*, 2010; Kastner *et al.*, 2011). No function was previously suggested for the low molecular weight LptpA1 and LptpA2. The PTP LptpB1/LipA was suggested to contribute to the virulence of two *Listeria* strains in a mouse infection model without obvious changes in macrophage or epithelial cells' growth curve assays (Kastner *et al.*, 2011). We obtained similar results in the cellular growth curves of the four PTP deletion mutant but did not observe major virulence defect in the *in vivo* mouse infection models using the 10403S WT strain.

To conclude, the major phenotype that we have observed associated with PTPs deletion in *L. monocytogenes* was changes in GlcNAc glycosylation of WTA. However, the precise role of the tyrosine phosphatases in the modification of this extracellular polysaccharide remains unclear. The fact that there are similar PTPs in other pathogenic bacteria emphasizes the importance of understanding the role of bacterial PTPs and tyrosine phosphorylation.

Acknowledgements

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Supplementary materials and methods.
Fig. S1. Sensitivity of *Listeria monocytogenes* strains to Lysozyme.
Fig. S2. Growth curve in activated macrophages.
Fig. S3. Virulence in mice.
Fig. S4. Analysis of total cell wall phosphate content of wild-type strain 10403S and deletion mutant DP-L5359.
Table S1. Primers used in this study.
Table S2. List of the sequenced *Listeria* spp. and strains available at the NCBI database in which BLAST P analysis was presented in Table 2.

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