Wall Teichoic Acids Restrict Access of Bacteriophage Endolysin Ply118, Ply511, and PlyP40 Cell Wall Binding Domains to the Listeria monocytogenes Peptidoglycan

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The C-terminal cell wall binding domains (CBDs) of phage endolysins direct the enzymes to their binding ligands on the bacterial cell wall with high affinity and specificity. The Listeria monocytogenes Ply118, Ply511, and PlyP40 endolysins feature related CBDs which recognize the directly cross-linked peptidoglycan backbone structure of Listeria. However, decoration with fluorescently labeled CBDs primarily occurs at the poles and septal regions of the rod-shaped cells. To elucidate the potential role of secondary cell wall-associated carbohydrates such as the abundant wall teichoic acid (WTA) on this phenomenon, we investigated CBD binding using L. monocytogenes serovar 1/2 and 4 cells deficient in WTA. Mutants were obtained by deletion of two redundant tagO homologues, whose products catalyze synthesis of the WTA linkage unit. While inactivation of either tagO1 (EGDe lmo0959) or tagO2 (EGDe lmo2519) alone did not affect WTA content, removal of both alleles following conditional complementation yielded WTA-deficient Listeria cells. Substitution of tagO from an isopropyl-β-D-thiogalactopyranoside-inducible single-copy integration vector restored the original phenotype. Although WTA-deficient cells are viable, they featured severe growth inhibition and an unusual coccoid morphology. In contrast to CBDs from other Listeria phage endolysins which directly utilize WTA as binding ligand, the data presented here show that WTAs are not required for attachment of CBD118, CBD511, and CBDP40. Instead, lack of the cell wall polymers enables unrestricted spatial access of CBDs to the cell wall surface, indicating that the abundant WTA can negatively regulate sidewall localization of the cell wall binding domains.

Bacteriophage endolysins are cell wall-hydrolyzing enzymes produced during the late phase of gene expression in the lytic cycle of virus multiplication, mediating the release of progeny phages (3, 32). They are usually composed of two functional domains, an enzymatically active domain (EAD) at the N terminus and a cell wall binding domain (CBD) at the C-terminal part of the protein. Although endolysins have attracted attention as perspective tools and antimicrobial agents (3, 17, 21, 32), surprisingly little is known about the identity and structure of their cell wall-associated CBD binding ligands.

The cell envelope of a typical Gram-positive organism is composed of peptidoglycan, proteins, and secondary cell wall polymers, including teichoic acids (TAs) and other glycopolymers. Peptidoglycan together with the attached polymer chains plays a crucial role in bacterial physiology and assumes a variety of other functions (28, 35, 54). TAs represent the most abundant polymer associated with the cell walls of Gram-positive bacteria and include cell wall-anchored teichoic acids (WTAs) and membrane-anchored lipoteichoic acids (LTAs), differing in synthesis and chemical structure (37). WTAs are phosphate-containing, anionic carbohydrate polymers covalently attached to the peptidoglycan via a conserved linkage unit (37, 52). Although their structures may be highly variable between species or even strains (54), the repeating units of the WTA polymers often feature glyceral phosphate (e.g., in Bacillus subtilis 168) or ribitol phosphate (e.g., in Staphylococcus aureus and Listeria monocytogenes) backbones (56) and are often modified and substituted with different sugars or amino acids, such as β-alanine (27, 37). LTAs usually show less structural diversity (54). In L. monocytogenes, LTA polymers consist of hydrophilic poly(glyceral phosphate) chains decorated with β-alanine and galactosyl residues (23, 39, 50).

The CBD of Listeria phage endolysin PlyP35 specifically recognizes N-acetylgalcosamine (GlcNAc) residues attached to the ribitol phosphate in WTA polymers of L. monocytogenes serovar 1/2 and 3 strains (11). In contrast, the CBDs of Listeria phage endolysins Ply118, Ply511, and PlyP40 (34, 43) investigated here are assumed to recognize a more common, serovar-independent motif in the peptidoglycan structure (43). Green fluorescent protein (GFP)-tagged CBD118, CBD511, and CBDP40 molecules mainly attach to poles and septal regions of Listeria cells (33, 43), while the nature of the binding ligands remains unclear. The specific spatial distribution of binding ligands on the Listeria cell surface could also suggest that CBD targeting might be regulated and/or affected by the presence of other components in the cell envelope, such as the extremely abundant WTA polymers, which were reported to constitute up to 70% of the Listeria cell wall dry weight (13, 14).

Although WTA polymers are not essential for viability in B. subtilis and S. aureus, they contribute to host-cell binding, immune evasion, and virulence properties (8, 53, 54, 56). B. subtilis and S. aureus cells devoid of WTAs have been obtained by inactivation of tagO, whose product initiates the first step in WTA synthesis, transfer of GlcNAc phosphate to its lipid carrier, undecaprenyl phosphate (45, 53). The situation in L. monocytogenes had not been investigated, until now. Therefore, the aim of this study was to define the ligands in the bacterial cell envelope responsible for attachment of the CBDs to Listeria cells.
for binding of CBD118, CBD511, and CBDP40 and to explain the uneven spatial distribution of CBD molecules on *Listeria* cell walls. Toward this goal, we identified the key genes involved in WTA synthesis, which allowed us to construct WTA-deficient mutants in order to assess CBD binding and the role of WTA in this process.

**MATERIALS AND METHODS**

**Bacteria, plasmids, and growth conditions.** The strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were cultured aerobically at 37°C in Luria-Bertani (LB) medium with shaking and used for cloning, amplification of plasmids, and recombinant protein synthesis. *Listeria monocytogenes* strains were grown in brain heart infusion (BHI) medium or tryptose broth (TB) at 30°C with shaking. The following antibiotics were added to broth as selective agents when appropriate: ampicillin (100 μg/ml for *E. coli*), chloramphenicol (10 μg/ml for *E. coli* and *Listeria monocytogenes*), erythromycin (300 μg/ml for *E. coli*), 10 μg/ml for *Listeria monocytogenes*), and tetracycline (30 μg/ml for *E. coli*) (Sigma-Aldrich, Buchs SG, Switzerland). Media were supplemented with IPTG (isospropl-β-D-thiogalactopyranoside; 240 μg/ml; Carl Roth GmbH, Karlsruhe, Germany) for IPTG-inducible gene expression, if applicable. The plasmids used were purchased from Fermentas (Le Mont-sur-Lausanne, Switzerland). DNA fragments and PCR products used for cloning and construction of plasmids were created with Phusion high-fidelity DNA polymerase (Finnzymes, Espoo, Finland). PCR products and DNA restriction fragments were purified with a GenElute PCR cleanup kit (Sigma-Aldrich). Plasmids were purified using a GenElute plasmid miniprep kit (Sigma-Aldrich). Restriction enzymes and T4 DNA ligase (Roche Diagnostics, Rotkreuz, Switzerland) were used according to the manufacturer’s protocols. DNase, RNase, and proteinase K were purchased from Fermentas (Le Mont-sur-Lausanne, Switzerland).

**Binding of Endolysins to Listeria Cell Wall**

**TABLE 1** Bacterial strains and plasmids 

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Reference or origin</th>
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<tr>
<td><strong>Listeria monocytogenes strains</strong></td>
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<tr>
<td>EGDe</td>
<td>Wild type; serovar 1/2a</td>
<td>J. Kreft</td>
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<tr>
<td>WSLC 1042</td>
<td>Wild type; serovar 4b</td>
<td>ATCC 23074</td>
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<tr>
<td>EGDeΔtagO1</td>
<td>EGDeΔlmo0959; EGDe carrying a chromosomal deletion of <em>lmo0959</em></td>
<td>This study</td>
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<tr>
<td>EGDeΔtagO2</td>
<td>EGDeΔlmo2519; EGDe carrying a chromosomal deletion of <em>lmo2519</em></td>
<td>This study</td>
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<td>1042ΔtagO1</td>
<td>1042ΔLMO1042_0979; WSLC 1042, carrying a chromosomal deletion of <em>LMO1042_0979</em></td>
<td>This study</td>
</tr>
<tr>
<td>1042ΔtagO2</td>
<td>1042ΔLMO1042_2492; WSLC 1042, carrying a chromosomal deletion of <em>LMO1042_2492</em></td>
<td>This study</td>
</tr>
<tr>
<td>EGDeΔtagO1ΔtagO2::pLIV2(tagO1)</td>
<td>EGDe with a double deletion (Δlmo0959 and Δlmo2519) and lmo0959 expressed from an IPTG-inducible promoter; Cam' IPTG (+/−)</td>
<td>This study</td>
</tr>
<tr>
<td>1042ΔtagO1ΔtagO2::pLIV2(tagO1)</td>
<td>WSLC 1042 with a double deletion (ΔLMO1042_0979 and ΔLMO1042_2492) and LMO1042_0979 expressed from an IPTG-inducible promoter; Cam' IPTG (+/−)</td>
<td>This study</td>
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| **Escherichia coli strains** | | |
| XL-1 Blue MRF | Used for plasmid manipulations; ΔmcrA183 Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac [F' proAB lacP2ΔM15 Tn10 (Tet')] | Stratagene |
| XL-1 Blue MRF pHGFP_CBD118 | Used for protein expression of HGFP_CBD118; Amp' Tetr' | 33 |
| XL-1 Blue MRF pHGFP_CBD511 | Used for protein expression of HGFP_CBD511; Amp' Tetr' | 43 |
| XL-1 Blue MRF pHGFP_CBDP40 | Used for protein expression of HGFP_CBDP40; Amp' Tetr' | 43 |
| XL-1 Blue MRF pHGFP_CBDP35 | Used for protein expression of HGFP_CBDP35; Amp' Tetr' | 43 |

| **Plasmids** | | |
| pKS7 | Gram-negative bacterium/Gram-positive bacterium shuttle vector; thermosensitive; 6.9 kb; Cam' | 44 |
| pAUL-A | Gram-negative bacterium/Gram-positive bacterium shuttle vector; thermosensitive; 9.2 kb; Ery' | 5 |
| pLIV2 | *L. monocytogenes* site-specific phage integration vector; IPTG-controlled expression; 6.1 kb; Cam' | 24 |
| pKS7Δlmo0959 | pKS7 with lmo0959 flanking regions; Cam' | This study |
| pKS7Δlmo2519 | pKS7 with lmo2519 flanking regions; Cam' | This study |
| pKS7ΔLMO1042_0979 | pKS7 with LMO1042_0979 flanking regions; Cam' | This study |
| pKS7ΔLMO1042_2492 | pKS7 with LMO1042_2492 flanking regions; Cam' | This study |
| pAUL-AΔlmo0959 | pAUL-A with lmo0959 flanking regions; Ery' | This study |
| pAUL-AΔLMO1042_0979 | pAUL-A with LMO1042_0979 flanking regions; Ery' | This study |
| pLIV2Δlmo0959 | pLIV2 with lmo0959 under IPTG control; Cam' | This study |
| pLIV2ΔLMO1042_0979 | pLIV2 with LMO1042_0979 under IPTG control; Cam' | This study |

a. Genes *lmo0959* and *LMO1042_0979* were designated tagO1; genes *lmo2519* and *LMO1042_2492* were named tagO2.

b. IPTG (+/−). IPTG was added when required to regulate the expression of tagO1 under the control of the inducible P*phaC* promoter.
were constructed by splicing by overlap extension PCR (SOE-PCR) (25). Genes of *L. monocytogenes* serovar 4b strain WSLC 1042 were named according to the annotated genome sequences of *L. monocytogenes* serovar 4b strain F2365. The SOE-PCR primers listed in Table S1 in the supplemental material were designed to amplify upstream (primer pair A and B) and downstream (primer pair C and D) fragments of relevant genes by using EGDe or WSLC 1042 chromosomal DNA as the template. Fragments with in-frame deletions were then generated in an SOE-PCR using products AB and CD and the external primers A and D. The resulting products (AD) were digested with SacI and BamHI and ligated into the corresponding sites of the temperature-sensitive shuttle vector pKS7 (44). The constructs were transformed into *E. coli* strain XL1-Blue MRF*, resulting in pKS7(Vlm0095) and pKS7(Vlm0097), used for strain EGDe, as well as pKS7(ΔMO1042_2492) and pKS7(ΔMO1042_0879), used for strain WSLC 1042.

Isolated plasmids were then electroporated into competent *L. monocytogenes* EGDe or WSLC 1042 using a previously published protocol (38). On the basis of the temperature-sensitive origin of replication of pKS7, chromosomal integration was selected at 42°C (nonpermissive temperature) in the presence of antibiotics. After a series of passages and growth at the permissive temperature (30°C) in the absence of selective drugs, deletion of target genes was obtained via homologous recombination and allelic replacement (44). Colonies were screened for the loss of antibiotic resistance. Antibiotic-sensitive mutants EGDεΔtagO1 and EGDεΔtagO2, as well as 1042ΔtagO1 and 1042ΔtagO2, were confirmed by PCR and DNA sequencing to verify in-frame deletions.

**Inducible expression of tagO.** *L. monocytogenes* tagO1 genes lmo0959 (strain EGDε) and LMO1042_0979 (strain WSLC 1042), including their native ribosome binding sites, were amplified from chromosomal DNA using the primer pairs listed in Table S1 in the supplemental material. PCR products were digested with BamHI and PstI and inserted into pLIV2 (24) immediately downstream of the IPTG-inducible Pspac promoter. pLIV2 is an integrative shuttle plasmid derived from pLIV1 (6) and the site-specific integration vector pPL2 (30). The constructs pLIV2(Vlm0095) and pLIV2(Vlm0097) were electroporated into *E. coli* strain XL1-Blue MRF*, and purified plasmids were used as described below.

**Conditional ΔtagO1 ΔtagO2 double-deletion mutants.** Our initial attempts to delete both tagO alleles sequentially failed, since mutant bacteria were apparently not viable. In an alternative approach, we constructed conditional mutants using pLIV2(Δlmo0959) and pLIV2(Δlmo0979), to enable regulated expression of tagO under the control of the inducible promoter Ppuc. Vector pLIV2(Δlmo0959) was introduced into EGDεΔtagO1, followed by selection for plasmid integration on BHI plates supplemented with chloramphenicol, yielding strain EGDεΔtagO1::pLIV2(Δlmo0959). Similarly, plasmid pLIV2(Δlmo0979) was used to generate strain 1042ΔtagO1::pLIV2(Δlmo0979). In the next step, conditional ΔtagO1 ΔtagO2 double-deletion mutants were constructed by allelic replacement using the temperature-sensitive shuttle vector pAUL-A (5) containing an erythromycin resistance marker. SOE-PCR products of lmo0959 and LMO1042_0979 digested with SacI and BamHI were cloned into pAUL-A, yielding pAUL-A(Δlmo0959) and pAUL-A(ΔLMO1042_0979).

After transformation into *Listeria*, insertion mutants were generated according to the procedure described above. Excision of chromosomally integrated plasmids was performed by repeated growth at 30°C in the absence of erythromycin but in the presence of chloramphenicol and 1 mM IPTG to maintain the plasmid copy number. After 1 to 2 days of growth, cells were collected by centrifugation (20,000 × g for 5 min), and cell walls were recovered by centrifugation (3,000 × g, 10 min, 4°C), and resuspended in SM buffer (50 mM Tris HCl, 100 mM NaCl, 8 mM MgSO4, pH 7.5). Cells were broken by two passages through a One-Shot cell disrupter (Constant cell disruption system; Northants, United Kingdom) at a pressure of 270 MPa. Larger debris and remaining intact cells were removed by centrifugation at 1,500 × g for 5 min. Cell walls were recovered by centrifugation of the supernatant (20,000 × g, 30 min, 4°C). The cell wall-containing pellet was washed three times with water and resuspended in SM buffer. DNase/RNase (25°C, 4 h) and proteinase K (25°C, 2 h) were added at a concentration of 100 μg/g cell wall wet weight. Then, the same volume of 8% (wt/vol) boiling SDS was added, followed by further incubation for 30 min at 100°C to remove noncovalently associated components such as membranes, proteins, and LTA. SDS-insoluble material was collected by centrifugation (20,000 × g, 30 min, 20°C). Finally, the cell wall-containing pellet was washed five times in pure water by repeated centrifugation and resuspension, lyophilized, and stored at −20°C.

**Determination of cell wall-associated phosphate.** To determine the relative amount of WTA in cell walls of wild-type and mutant strains, lyophilized purified cell walls (i.e., murine and covalently attached WTA) were assayed for total (cell wall-bound) phosphate as described elsewhere (12). Briefly, 0.1 mg purified peptidoglycan dissolved in 5 ml distilled water was treated with decomposition reagent (NANOCOLOR NanOx metal; Macherey-Nagel, Switzerland) for oxidative decomposition of cell walls according to the manufacturer’s protocol. Subsequently, total phosphorus was determined using a photometric phosphate test (Spectrophotophosphate; Merck, Zug, Switzerland).

**CBD binding and fluorescence microscopy.** GFP-tagged CBD proteins derived from phage endolysins P118, P311, P404, and P403 were produced in *E. coli* and purified by affinity chromatography as described earlier (33, 43). Protein concentration was determined using a NanoDrop ND-1000 analyzer, and purity was checked by SDS-PAGE analysis. Binding of GFP-CBD to bacteria was tested as described before (33). Cells were grown overnight in BHI medium (30°C with shaking) supplemented with antibiotics and IPTG as indicated. Then, cultures were diluted 10-fold into fresh medium and incubated for another 2 h. In case of the conditional double-deletion mutants, overnight cultures were sedimented by centrifugation, washed once with prewarmed BHI, resuspended in fresh medium, and used to inoculate growth medium without IPTG. Bacterial samples (2 to 5 ml) were collected in the late log phase (centrifugation at 7,000 × g for 1 min), washed with phosphate-buffered saline–Twee 20 (PBS-T) buffer (50 mM NaH2PO4, 120 mM NaCl, 0.1% Tween 20, pH 8.0), and resuspended in 1/10 volume PBS-T. For binding assays, 100 μl of the resuspended cells was mixed with 50 μl of an 800 nM solution of the GFP-CBD proteins, and the mixture was incubated for 5 min at room temperature. Cells and bound protein were pelleted at 16,000 × g for 1 min, resuspended and washed twice in 500 μl PBS-T, and finally, resuspended in 50 μl PBS-T and analyzed by confocal laser scanning fluorescence microscopy (Leica TCS SPE; Leica, Heerbrugg, Switzerland) using GFP-specific excitation and emission wavelengths.

To permeabilize and remove the outer membrane of *E. coli* cells for
determination of CBD decoration of the A1g peptidoglycan of Gram-negative bacteria, cells were treated as described previously (31). Briefly, exponentially growing E. coli cells were centrifuged (7,000 × g, 5 min, 10°C), resuspended in chloroform-saturated 50 mM Tris buffer (pH 7.7), and incubated for 45 min at room temperature. Finally, cells were washed to remove the solvent, adjusted to an OD_{600} of 1.0, and used for the CBD binding assays described above.

RESULTS

Two homologous tagO genes in L. monocytogenes. The tagO products from B. subtilis and S. aureus have been shown to catalyze the first step in WTA biosynthesis, the transfer of GlcNAc to the bactoprenol carrier (45, 53). To provide a basis for the construction and analysis of WTA-deficient Listeria mutants, the first aim was to identify tagO homologues in the genomes of L. monocytogenes EGDe (serovar 1/2a) and F2365 (serovar 4b). Surprisingly, in silico analysis indicated two separate tagO orthologues with highly similar amino acid sequences (53% identity, 74% similarity) in the genomes of L. monocytogenes serovar 1/2 strain EGDe (genes lmo0959 and lmo2519; GenBank accession numbers NP_464484 and NP_466042, respectively) and in L. monocytogenes serovar 4b strain F2365 (genes LMO2365_0979 and LMO2365_2492; GenBank accession numbersYP_013580 and YP_015080, respectively) (Fig. 1). The genes are predicted to encode putative UDP-GlcNACundecaprenyl phosphate GlcNAC-1-phosphate transferases. Since they reveal significant homologies to the PlyP40 (i.e., their CBDs), we constructed tagO deletion mutants in the parental strain (33).

Deletion of tagO in L. monocytogenes serovar 1/2a and 4b strains. To determine the role of WTA polymers for recognition and binding of Listeria phage endolysins Ply118, Ply511, and PlyP40 (i.e., their CBDs), we constructed tagO deletion mutants in strains representing the two different WTA backbone types occurring in L. monocytogenes (18). Functional inactivation of tagO is expected to disrupt the first step of the WTA linkage unit biosynthetic pathway (45). We found that mutants featuring removal of a single allele (EGDeΔtagO1, EGDeΔtagO2, 1042ΔtagO1, and 1042ΔtagO2) showed absolutely no defects in growth or morphology. They were identical to the parent strains. This indicated that growth, division, and viability were not affected by removal of only one of the two apparently functionally redundant tagO alleles.

As a next step, we aimed to generate mutants defective in both genes, i.e., ΔtagO1 ΔtagO2 double deletions. However, all our attempts employing the classical two-step allelic replacement strategy were unsuccessful; i.e., the second exchange to inactivate the remaining tagO allele in EGDeΔtagO1::pKS771(ΔtagO2) and EGDeΔtagO2::pKS771(ΔtagO1) or in 1042ΔtagO1::pKS771(ΔtagO2) and 1042ΔtagO2::pKS771(ΔtagO1) failed. This finding suggested that at least one functional tagO locus is required and essential for normal cell growth and viability of Listeria. As a solution to the problem, we constructed double-deletion mutants which additionally feature conditional, regulated expression of tagO1 from an integrated single-copy plasmid, which yielded strains EGDeΔtagO1ΔtagO2::pLIV2(tagO1) and 1042ΔtagO1ΔtagO2::pLIV2(tagO1). Both mutants feature full in-frame chromosomal deletion of both tagO genes and a functional tagO allele under the control of an IPTG-inducible promoter. Both strains were viable and used for further studies.

Deletion of tagO results in loss of WTA. To assess the impact of tagO deletion on WTA content in the Listeria peptidoglycan, the amount of phosphate (as a direct measure for WTA) present in purified cell walls of tagO single- and double-deletion mutants was determined and compared to that for the wild type. As shown in Fig. 2, deletion of only one of the two tagO alleles in strains EGDe and WSLC 1042 did not significantly affect the level of cell wall-associated phosphate in comparison to that for the wild type, confirming the functional redundancy of tagO1 and tagO2 in WTA biosynthesis. On the other hand, total phosphate associated with the cell walls of EGDeΔtagO1ΔtagO2::pLIV2(tagO1) and 1042ΔtagO1ΔtagO2::pLIV2(tagO1) double-deletion mutants grown in the absence of IPTG was drastically reduced, to approximately 30 to 35% of the wild-type level. In the presence of IPTG, total cell wall phosphate in the conditional double-deletion mutants was almost completely restored to about 85 to 90% of that of the parental strain (33).

The L. monocytogenes serovar 1/2 EGDe mutant cells were also tested for decoration by fluorescent CBDP35, which specifically recognizes and binds to terminal GlcNAc residues attached to the

FIG 1 Genome regions harboring tagO homologues in the chromosomes of different Listeria strains. tagO1 (A) and tagO2 (B) genes from Listeria strains of different species and serovars (SV) are shown: L. monocytogenes (Lmo) EGDe (serovar 1/2a), L. monocytogenes strain F2365 (serovar 4b), L. innocua (Lin) Clip 11262 (serovar 6a), L. seeligeri (Lse) SLCC 3954 (serovar 1/2b), and L. welshimeri (Lwe) SLCC 5334 (serovar 6b). The tagO genes are shown as solid black arrows, and the individual gene designations are listed below. They are located at approximately opposite positions on the bacterial chromosomes.
poly(ribitol phosphate) backbone of Listeria WTA (11). Single tagO1 or tagO2 deficiency did not affect binding of CBD35 (Table 2). In contrast, inactivation of both alleles in EGDeΔtagO1ΔtagO2:: pLIV2(tagO1) resulted in loss of decoration by CBD35 (Table 2). These findings correlate well with those of the chemical analysis described above and provide additional evidence for the drastically reduced WTA level in these cells.

WTA deficiency leads to impaired growth and cocoid cell morphology. Single deletion of the tagO locus in EGDeΔtagO1, EGDeΔtagO2, 1042ΔtagO1, and 1042ΔtagO2 did not affect cell morphology or growth properties compared to those of the wild type (Fig. 3). In contrast, inactivation of both alleles in EGDeΔtagO1ΔtagO2::pLIV2(tagO1) and 1042ΔtagO1ΔtagO2:: pLIV2(tagO1) was characterized by dramatic alterations in cell morphology (i.e., cocoid cells) and formation of aggregates when tagO expression was shut down following removal of IPTG. Additionally, WTA deficiency resulted in a substantially reduced growth rate, indicating a major role of WTA for normal cell growth and development. Transfer to IPTG-containing medium restored the normal phenotype (Fig. 2). These findings clearly indicate that the presence of at least one of the two redundant tagO homologues in Listeria is required for maintaining cell shape and growth response.

CBD118, CBD511, and CBDP40 do not require WTA for binding. To determine the potential role of the WTA polymers for binding of CBD118, CBD511, and CBDP40, tagO null mutants were tested for decoration by GFP-tagged CBDS (Table 2; Fig. 4). CBD511 and CBDP40 decorate the entire cell surface of wild-type EGDe and WSLC 1042, with a clear preference (i.e., more intensive decoration) for septal and polar regions (43). The same binding pattern was observed for mutant strains EGDeΔtagO1, EGDeΔtagO2, 1042ΔtagO1, and 1042ΔtagO2. Interestingly, binding of CBD511 and CBDP40 to the WTA-deficient double tagO null mutant was no longer restricted to poles and septa but occurred at a high density and was evenly distributed over the entire lateral (sidewall) cell surface.

Similar results were obtained for CBD118, which labeled the target cells at polar and septal regions (33). There was no difference in decoration of EGDe and the single-locus deletion mutants EGDeΔtagO1 and EGDeΔtagO2 (Table 2; Fig. 4). In contrast, CBD118 was found to decorate the entire cell surface of the WTA-deficient double-deletion mutant EGDeΔtagO1ΔtagO2:: pLIV2(tagO1). Binding to these cells appeared to be significantly stronger than that for the parental bacteria, as judged from fluorescence microscopy imaging. Moreover, WTA-deficient double-deletion mutants of serovar 4b strain WSLC 1042 were also found to be labeled by CBD118. This was a very surprising result, given the fact that CBD118 normally recognizes Listeria serovar 1/2 and 3 cells and does not bind to strains featuring serovar 4, 5, or 6 (33). Conditional double-deletion mutants grown in the presence of IPTG fully restored the wild-type CBD binding properties (Fig. 4).

The peptidoglycan of Listeria belongs to the meso-diaminopimelic acid (m-DAP) cross-linked A1γ chemotype, which is also featured by many Gram-negative bacteria, such as E. coli (42). In this context, the ability of CBD118, CBD511, and CBDP40 to decorate the peptidoglycan of E. coli cells stripped of their outer membrane (Fig. 5) further supports our conclusion that the different CBDS share the ability to recognize and bind to the A1γ peptidoglycan backbone.

Taken together, our findings demonstrate that CBD118, CBD511, and CBDP40 directly interact with the peptidoglycan...
backbone structure and that the WTA polymers present on *Listeria* cells are not required for this interaction. Moreover, their presence seems to negatively regulate access of the endolysin ligands for binding of the CBDs.

**DISCUSSION**

The mostly C-terminally located cell wall binding domains of endolysins from bacteriophages infecting Gram-positive bacterial host cells recognize specific peptidoglycan-associated ligands on the surface of the bacteria with high affinity and specificity. However, surprisingly few endolysin ligands have so far been identified. The Cpl-1 enzyme from pneumococcal phage Cp-1 and other pneumococcal endolysins use choline-binding modules for anchoring to choline-containing teichoic acids of the pneumococcal cell wall (19,21, 22). Further, the C terminus of endolysin Lyb5, encoded by *Lactobacillus fermentum* bacteriophage PYB5, has been shown to bind to the peptidoglycan, mediated by LysM
repeat regions (26). Regarding enzymes from Listeria phages, we recently reported that CBDP35 specifically recognizes terminal GlcNAc residues in the Listeria WTA molecules (11). In the work presented here, we show that the CBDs of Listeria phage endolysins Ply118, Ply511, and PlyP40 specifically interact with the peptidoglycan backbone structure of Listeria cells. In contrast to CBDP35, WTA polymers seem to be dispensable and are not required for recognition and binding. Instead, they apparently play a role in orchestrating the specific spatial localization of the endolysins to cell division sites and poles.

Use of GFP-tagged CBD fusion proteins to decorate cell walls of Listeria strains of different serovar types revealed individual binding partners of the different phage endolysins, which correlate very well with serovar- and strain-specific cell surface structures (33, 43). The binding patterns and properties of CBD118, CBD511, and CBDP40 differ fundamentally from those of other Listeria phage endolysin CBDs, such as CBD500, CBDPSA, and CBDP35 (29, 33, 43). The latter group of CBDs was found to recognize and bind to the entire cell surface, while CBD118, CBD511, and CBDP40 primarily associate with poles and septae (33, 43). Spatially distinct cell wall targeting has also been reported for other peptidoglycan hydrolases, such as Lactococcus phage endolysin Lyb5 (26), the AcmA autolysin of Lactococcus lactis (46), and the Atl autolysin of S. aureus (41). The uneven distribution of these enzymes over the cell wall is also proposed to be due to the presence of secondary cell wall polymers such as teichoic acids that prevent targeting to the entire cell surface (41, 47).

Cell walls of L. monocytogenes contain two different polyanionic polymers: WTA, which is covalently linked to the peptidoglycan via a linkage unit, and LTA, which is anchored to the cytoplasmic membrane (35, 37). WTA polymers can account for up to 60 to 70% of the total dry mass of isolated Listeria cell walls (13, 14). During late log phase of bacterial growth, WTA polymers are evenly distributed over the entire cell surface. This is nicely illustrated by using fluorescent CBDP35 (11, 43), which targets the terminal GlcNAc substituted to position C-4 of the poly(ribitol phosphate) WTA backbone (11). In light of the findings described here, we postulate that the asymmetric distribution of CBD118, CBD511, and CBDP40 to the sites of cell division may also be based on WTA polymers. However, the polymers do not serve as binding ligands but seem to prevent an even distribution of the cell wall binding proteins to the peptidoglycan sacculus. The hypothesis was then tested and confirmed using WTA-deficient Listeria cells.

TagO catalyzes the initial step in synthesis of the WTA linkage unit, i.e., the formation of undecaprenyl-diphospho-N-acetylglucosamine (45, 48, 53). In order to gain better insight into WTA biosynthesis in L. monocytogenes, we made use of the fact that the WTA linkage units in Gram-positive bacteria feature conserved structures (1, 53). Based on sequence homologies with B. subtilis and S. aureus TagO, we identified two different Listeria genes designated tagO1 and tagO2. As suggested earlier (56), the designation “tag” (teichoic acid glycerol) was used instead of “tar” (for teichoic acid ribitol), because of the highly conserved mechanisms in biosynthesis of the polymers. Our in silico and experimental analysis of L. monocytogenes TagO1 and TagO2 functions demonstrated their functional redundancy.

It was shown that TagO is not essential for cell viability in B. subtilis and S. aureus (8, 9), although mutants revealed growth and division defects. Along this line, deletion of lmo2537 in L. mono-
feature slightly different properties. These might be based on their similar but not identical amino acid sequences and on the existence of diverse and different peptidoglycan-binding motifs in the enzymes. CBDP40 has cell wall recognition motifs similar to bacterial SH3 and LysM motifs, whereas no such sequences could be identified in CBD118 and CBD511 (43). The peptidoglycan-binding properties of LysM domains appear to be dependent on other cell wall constituents, such as proteins, cell wall polymers, and even modifications of the peptidoglycan itself (4).

CBD118 offers the narrowest cell wall binding range, which correlates well with the host range of the parent A118 phage. This temperate phage infects Listeria serовар 1/2 strains only and does not recognize cells from serovars 4, 5, and 6 (33). Intriguingly, we show here that WTA-depleted serovar 4b cells (strain WSLC 1042) accept GFP-tagged CBD118 on the entire surface. This finding strongly suggests that the inability of CBD118 to target native serovar 4b cell walls is due to a mechanism of exclusion by the cell wall polymers.

In conclusion, the extremely abundant WTA polymers present on the Listeria cell surface assume crucial roles in the targeting and spatial distribution of phage endolysins CBD118, CBD511, and CBDP40 to septal and polar regions of the peptidoglycan. With increasing consideration of endolysins as potential antimicrobial agents and their cell wall binding domains as tools for immobilization and rapid detection of pathogens, molecular analysis of the endolysin and cell wall interactions becomes more and more important.

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