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Contribution of sortase A to the regulation of *Listeria monocytogenes* LPXTG surface proteins

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Summary. Gram-positive bacteria of the genus *Listeria* contain many surface proteins covalently bound to the peptidoglycan. In the pathogenic species *Listeria monocytogenes*, some of these surface proteins mediate adhesion and entry into host cells. Specialized enzymes called sortases anchor these proteins to the cell wall by a mechanism involving processing and covalent linkage to the peptidoglycan. How bacteria coordinate the production of sortases and their respective protein substrates is currently unknown. The present work investigated whether the functional status of the sortase influences the level at which its cognate substrates are produced. The relative amounts of surface proteins containing an LPXTG sorting motif recognized by sortase A (StrA) were determined in isogenic wild-type and Δ*srtA* strains of *L. monocytogenes*. The possibility of regulation at the transcriptional level was also examined. The results showed that the absence of SrtA did not affect the expression of any of the genes encoding LPXTG proteins. However, marked differences were found at the protein level for some substrates depending on the presence/absence of SrtA. In addition to the known "mis-sorting" of some LPXTG proteins caused by the absence of SrtA, the total amount of certain LPXTG protein species was lower in the Δ*srtA* mutant. These data suggested that the rate of synthesis and/or the stability of a subset of LPXTG proteins could be regulated post-transcriptionally depending on the functionality of SrtA. For some LPXTG proteins, the absence of SrtA resulted in only a partial loss of the protein that remained bound to the peptidoglycan, thus providing support for additional modes of cell-wall association in some members of the LPXTG surface protein family. [**Int Microbiol** 2012; 15(1):43-51]

Keywords: *Listeria monocytogenes* · sortases · peptidoglycan · surface proteins · covalent anchoring · bacterial regulation

Introduction

Gram-positive bacteria have a thick cell wall consisting of a multilayered peptidoglycan [26]. This backbone is decorated with teichoic (TA) and lipoteichoic acids (LTA) that are either directly bound to the peptidoglycan or tethered to the membrane, respectively. Besides these structures, the bacterial cell wall acts as a platform for the anchorage of a variety of surface proteins, many of which play essential physiological roles, for example, in nutrient acquisition, quorum sensing, biofilm formation, and host interactions [21]. The mechanisms of protein association with the cell wall are diverse and include non-covalent protein-peptidoglycan interactions via peptidoglycan-binding domains such as LysM and WxL; direct protein-TA and protein-LTA interactions; and covalent anchoring of the protein to the peptidoglycan lattice [1,21].

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In the latter case, the covalent association between the protein and the cell wall occurs upon recognition of the protein substrate by specialized enzymes called sortases [10,21]. Enzymatic cleavage of a C-terminal sorting motif in turn leads to the covalent anchorage of the protein to the peptidoglycan via a transpeptidation reaction. The first sorting motif, LPXTG, was identified in protein A of *Staphylococcus aureus*. Since then, distinct sortases with different specificities for different sorting motifs have been described [11,18, 24,30].

Genome sequencing studies have revealed that bacteria of the genus *Listeria* contain the largest family of genes encoding predicted surface proteins recognized by sortases. More than 40 genes of this class have been annotated in every *Listeria* genome sequenced to date [5,6,15,16]. *Listeria* contains two sortases, SrtA and SrtB, which recognize LPXTG and NPKSS/NAKTN sorting motifs, respectively [2,3, 20,27]. In the case of the pathogenic species *L. monocytogenes*, SrtA was reported to be essential for virulence [3]. This phenotype is linked to the major role in virulence played by distinct surface proteins containing the LPXTG sorting motif. Examples of these proteins are internalin A (InlA), Vip, InlJ and LapB, which promote bacterial adhesion and invasion of eukaryotic cells and modulate host immune responses [7,12,25,28,29]. By contrast, no evidence of a requirement for alternative sortase B in *L. monocytogenes* virulence has been found, and SrtB recognizes only the surface proteins Lmo2185 (SvpA) and Lmo2186 (SvpB) [2].

Our previous proteomic studies revealed that the absence of SrtA resulted in a cell-wall proteome devoid of the 13 LPXTG surface proteins that were detected by this technique in the wild-type strain [27]. This study was performed with peptidoglycan material obtained under harsh purification conditions, i.e., extensive boiling of the cell-wall extract in solutions containing high percentage of ionic detergents (4 % SDS). This methodology could have impacted, at least to some extent, the ability to detect LPXTG surface proteins that, in the absence of SrtA, were retained in the cell wall by non-covalent associations with other proteins or cell-wall components. These hypothetical cell-wall–protein associations might naturally play additional roles in the fine-tuning of protein function. A similar diversity of associations for a single surface protein with the cell wall was recently demonstrated for the *L. monocytogenes* ActA protein, involved in actin-tail polymerization in intracellular bacteria [13]. Thus, in addition to being tethered to the membrane by its C-terminal hydrophobic domain, ActA strongly associates with the cell wall in bacteria that proliferate within epithelial cells [13,14]. Variable associations of surface proteins with the cell wall have also been reported for members of the same protein family. Specifically, *L. monocytogenes* surface proteins covalently bound to the peptidoglycan by SrtA, such as InlA, InlH and InlJ, or by alternative sortase SrtB, such as Lmo2185 (SvpA), were recently shown to preferentially locate in distinct regions of the bacterial surface [4]. Another aspect, poorly understood, is to what extent, if any, sortases contribute to regulating the production of surface proteins that they recognize for anchoring to peptidoglycan. Consistent with what is known for essentially every enzymatic process, a tight co-regulation of the relative amount of the sorting enzyme and its substrates can be expected. Based on these considerations, in the present study the effect of a lack of *L. monocytogenes* SrtA on the expression of LPXTG surface proteins was examined. In addition, we analyzed whether the absence of SrtA results in changes in both the relative amounts and the subcellular distribution of these proteins.

Materials and methods

Bacterial strains and growth conditions. The *Listeria monocytogenes* serotype 1/2a strain used in this study was EGDe; its genome has been sequenced [15]. The isogenic mutant derivative was the previously described Δ*srtA* BUG 1777 [3]. Both were grown at 37 ºC to exponential phase (OD_{600} = 0.2) in brain-heart infusion (BHI) medium with shaking (150 rpm). For experiments involving stationary-phase regulation, the bacteria were grown at 37 ºC in BHI medium under static, non-shaking conditions. In these stationary-phase conditions, bacteria grown display high invasion rates when exposed to cultured eukaryotic cell lines [13].

Bacterial fractionation and Western blot analysis. Fractions containing cytosolic, membrane, cell-wall, and secreted proteins of *L. monocytogenes* grown at 37 ºC in BHI media were obtained as described previously [27], except that the pellets were incubated in lysis buffer for 4 h instead of 1 h. The supernatants, corresponding to the cell-wall fractions, were filtered and precipitated on ice in 15 % TCA for 1 h. Proteins present in the cell-wall fraction were recovered by centrifugation at 29,000 ×*g* for 20 min at 4 ºC . The pellet was washed in cold acetone and centrifuged again under the same conditions. A volume of the cell-wall fraction was analyzed by SDS-PAGE followed by Western blotting using polyclonal rabbit immune sera recognizing the LPXTG proteins Lmo0130, Lmo0159, Lmo0160, Lmo0263 (InlH), Lmo0610, and Lmo0880, and mouse monoclonal antibody anti-Lmo0434 (InlA). Other proteins analyzed in these fractions as controls were the StrB substrate SvpA and the peptidoglycan hydrolase Iap (P60). Polyclonal rabbit antibody recognizing P60 was a gift of Dr. Andreas Bubert (University of Würzburg, Germany). Mouse monoclonal anti-Lmo0434 (InlA) and polyclonal rabbit anti-SvpA were a gift of Prof. Pascale Cossart (Institut Pasteur, Paris, France).

RNA preparation and RT-PCR assays. RNA was purified in three independent experiments from bacteria grown in 10 mL of BHI at exponential phase (OD_{600} = 0.2). Total RNA from bacterial pellets was extracted

using the TRIzol reagent method (Invitrogen) [31]. RNA was treated with DNase I for 30 min at 37 ºC (Turbo DNA-free kit, Ambion/Applied Biosystems). RNA integrity and concentration were assessed by agarose-TAE electrophoresis and absorbance at 260 nm, respectively. RT-PCR was performed using a one-step RT-PCR kit (Qiagen). Briefly, RNA samples were diluted to 25 ng/µl and used in the RT-PCR, which was carried out in a final volume of 25 µl consisting of 5 µl buffer (5×), 1 µl of dNTP (10 mM), 3 µl each of the forward and reverse primers (5 mM), 1 µl of RT-PCR enzyme mix, 40 ng of RNA, and RNase-free water. RT-PCR cycling conditions were as follows: 50 ºC for 35 min and 95 ºC for 15 min, followed by 30 cycles of 94 ºC for 30 s, 55 ºC for 30 s, and 72 ºC for 40 s and then an extra elongation step at 72 ºC for 10 min. Oligonucleotides used in these RT-PCR assays were designed using the software Primer Express v3.0 (Applied Biosystems) and are listed in Table 1.

Results and Discussion

The expression of *L. monocytogenes* genes encoding LPXTG surface proteins is unaltered in a strain lacking sortase A. Sortases anchor surface proteins to the peptidoglycan of gram-positive bacteria once most of the protein substrate has passed through the membrane and is further retained there by its C-terminal region rich in hydrophobic amino acids [21]. At this stage, the sorting motif of the substrate protein is exposed on the outer leaflet of the membrane for recognition and cleavage by the sortase, leading to the covalent anchoring of the protein substrate to the peptidoglycan. Considering that the encounter between enzyme and substrate must be finely modulated spatially, temporally, and stoichiometrically, we examined whether the relative amounts of the sortase and its substrates were, to some extent, co-regulated. This type of regulation would prevent the potentially detrimental accumulation of surface proteins in their precursor form, i.e., tethered to the membrane, when either the level or the activity of SrtA was not optimal. This hypothesis was tested using the intracellular bacterial pathogen *L. monocytogenes*, which contains a large family of surface proteins bearing the LPXTG motif, recognized by StrA. Our first goal was to determine whether the expression of any of the genes encoding thesetranscriptionally regulated any of the genes encoding these LPXTG surface proteins could be modulated by StrA. Thus, the expression of the respective genes was analyzed by RT-PCR using total RNA purified from exponential cultures of the *L. monocytogenes* isogenic strains EGDe (wild-type) and BUG1777 (Δ*srtA*). The genome of the EGDe strain has been sequenced and is known to contain 41 LPXTG-encoding genes [15]. As expected, the relative expression levels of these genes in the wild-type strain varied, with some genes more highly expressed than others (Fig. 1). However, there were no major differences in the relative amounts of their respective transcripts between the wild-type and Δ*srtA* isogenic strains (Fig. 1). The only differences between the wild-type and *srtA* strains were in the relative levels of the *lmo0263* (*inlH*), *lmo0880*, *lmo1289* and *lmo1666* (*lapB*) transcripts (Fig. 1).

Further expression analyses by quantitative real-time PCR (qRT-PCR), however, ruled out the differential expression of any of these four genes (data not shown). Altogether, these data led us to conclude that the presence of SrtA on the *L. monocytogenes* cell surface does not, per se, constitute a cellular signal to modulate the expression of the genes encoding its protein substrates. Rather, as sugggested in previous reports [7,9,28,29], regulation appears to be governed mostly at the level of regulators that modulate the expression of a specific set of genes encoding the LPXTG surface proteins. Representative examples of these genes are *lmo0434 (inlA)*, regulated by PrfA and SigB [23], and *lmo0263* (*inlH*), *lmo0610*, *lmo0880* and *lmo2085*, regulated by SigB [9,17,31].

The lack of SrtA does not completely abolish the strong attachment of certain LPXTG surface proteins to cell-wall peptidoglycan. Since SrtA does not seem to regulate the expression of any of the 41 genes encoding the protein substrates of this sorting enzyme, we asked whether post-transcriptional regulatory mechanisms relied on the functional status of the sortase. To answer this question, it was necessary to quantify the relative levels of LPXTG proteins in the absence/presence of SrtA. Antibodies for all LPXTG surface proteins encoded in the genome of *L. monocytogenes* strain EGDe were generated, with the exception of anti-InlA and anti-InlJ sera, available from other sources. While all the immune sera recognized the recombinant protein produced for immunization purposes, only some of these sera readily recognized native LPXTG proteins in cell-wall extracts of *L. monocytogenes* (data not shown). In most cases, the latter consisted of those LPXTG proteins encoded by highly expressed genes (Fig. 1 and data not shown), such as InlA, Lmo0130, Lmo0160, Lmo0263 (InlH) and Lmo0610. In the other cases, differences were observed between the RT-PCR and Western-blot data, perhaps reflecting the existence of post-transcriptional regulatory mechanisms. Alternatively, and irrespective of the amount of protein produced, a distinct topology of the protein within the cell wall could render it either more resistant or labile to extraction from the peptidoglycan lattice.

Table 1. Oligonucleotides used in this study[a]

Oligonucleotide	Forward sequence (5′→3′)	Reverse sequence (5′→3′)
iap[b]	AAAGCAACTATCGCGGCTAC	TCTTGAACAGAAACACCGTA
lmo2026	CAGCCTCCACTTCATGGATTG	TCACCATGGGAAACTCCTGTAA
lmo2396	CCAGTTACCGGCCTAATTAAAGATT	TGGTATGCAGGATTGGACCAT
inlJ	GCAGCAACGAATGATGTTATTGATA	GCTAAGATCAAGGGTGGTAATGTTGT
lmo0171	ATCATCCAGTGAAGAAGCTGCAA	GTGAGCTCATTGTTGTTCACATTG
lmo0732	ACCAAAAACGGTCGACGTAAA	AGGTATTTGCCCAACCAAACG
lmo0175	TACGGACTTACAGGTTTCTGTTGGT	GCTTTAACAACTGGTGCGCTAA
lmo0327	CAATAACTGCCTTGCGAGTAACA	TGGAACTTTCACTAAGCGGTTATTATTT
inlI	CTTGGTTATTTAGCGCCTTTTGA	CCTGTTGCAGCCGCATTT
lmo0835	CAGATGGAGCGGAGATTGCT	TATGCAAGCCATCTATGCTAATGTC
lmo1413	GGCAATGCAGCTTATGTGCTT	AGTTTCACCGAGTTTTCCGGATA
lmo2178	GACTATGGATTCTCTTTTACCTGGTGAT	TTCCCTGAAAATGCAAAGTTCA
lmo2179	TTGAATCTGGTCAGACGGTAAACTAT	CTGCGCTTAATTCACCTGTTACTACT
inlA	AATATTAGTATTTGGCAGCGGAGTATG	GGCGTTATGTCCGTAAGTTGATT
lmo0130	GCGACAACTGACAATGCTATCC	GCCTTCTGCGACTGTGTTTG
lmo0159	CGATCTCGCTTTTGACGTGAA	TTACTAGCATCTACCCAACCATATTTGA
lmo0160	AGGGCAATTAACAGGCGATAAC	GACGCATCCAACACGTATCCT
inlG	AGTAGATAAAATGCCGGCTACGA	TTTCCGGGAGCTGTTTGAGA
inlH	CAGTAGCGCCAACGAAGGAT	GCGTCGCTGTTCCTAGCAA
inlE	GGTGAGCTTATTGCACCGGATA	TGCGTCATACCATCCATCGA
lmo0320	CCGTTTATCATCCAAGTGGCTAT	GCGCATCCAATTGTTGTTGA
lmo0331	GCCACCGTAACAAGCAGCTTAC	ACCTTGGCTTCGTGCATCTAG
inlF	ACCGAACTTGAAGTGGTCTTTACC	ATTTGCCCGGTTGTGAAGTC
lmo0435	GACTTTGTGGATGTTAGTGCGAAT	TGCTACGTCCTCCCCGTTT
lmo0463	GAGTTCAAGCTAGTCAAACAGTGGTT	CCAAATAAGGACGAGCACTAAGC
lmo0514	TGCTGCAGGACTCAAAGCAA	TGTCCACTGTCGCTTGTAGTCA
lmo0550	GCGTCGCCATTTTATGTGAA	ATGAAACCAACCCCTAGTAAAACAA
lmo0610	GTTTAAAAGCAACGCCAACACA	GTGGCGTCGGAGGTTCATT
lmo0627	TCACAAACCCAGTTGACATTCC	AATTTGCCGCGCCAGAA
lmo0725	TGCTGTTTTCATTTCACTTGGATT	GCAAGCGCGACAAGTACCA
lmo0801	AGATCCTGCCATGGCTAATGAA	TTAGCTAAATCAGCCACTGGTGAT
lmo0842	AACGGATGTGTCAGTGGGAGTATA	CCAGCCACGCTTAAGAAGGT
lmo0880	TAAAGTGCGAGTGGCGTATGA	TTTACCGGAAATGCGATTGG
lmo1115	ACGAAAGCTGGAGAAGAGGCTAA	AGAGAACGGAATAGGGCGTGTA
lmo1136	GCAACATCAACTACACTCGAGACA	AATTGTTACGGGCAAAGCGTAT
lmo1289	CTGAAATCAAAGCCACAACCAA	TAGGATTTCTTGAGTTTGAATGTTGAAC
lmo1290	ACAGGCATTGAATATGCTCACAATA	TGGCATTGTTTTAAGTGGCATAA
lmo1666	TTATGACTTCGACTGCTGACGAAA	TCGGACTTCCAACATCAACTACA
lmo1799	GATGATGGTAGCGCCTGTCA	CCGCCAATTCCTAAATAAGCAA
lmo2085	GTATTCAGCAAGATAGCGAAGAACCT	TCGTCGTTATTCCCGCATCTA
lmo2576	TCGTCGTTTACAATTGGTCGAT	CGCTGATTGGGAAAACGATT
lmo2714	CCGGCAGATGAAAACTTTGG	GTCACCTGTGCTTGGCAAATC

[a]Oligonucleotides were designed using the Primer Express v3.0 software (Applied Biosystems).
[b]Oligonucleotide sequences taken from the study of Cabanes et al. [7].

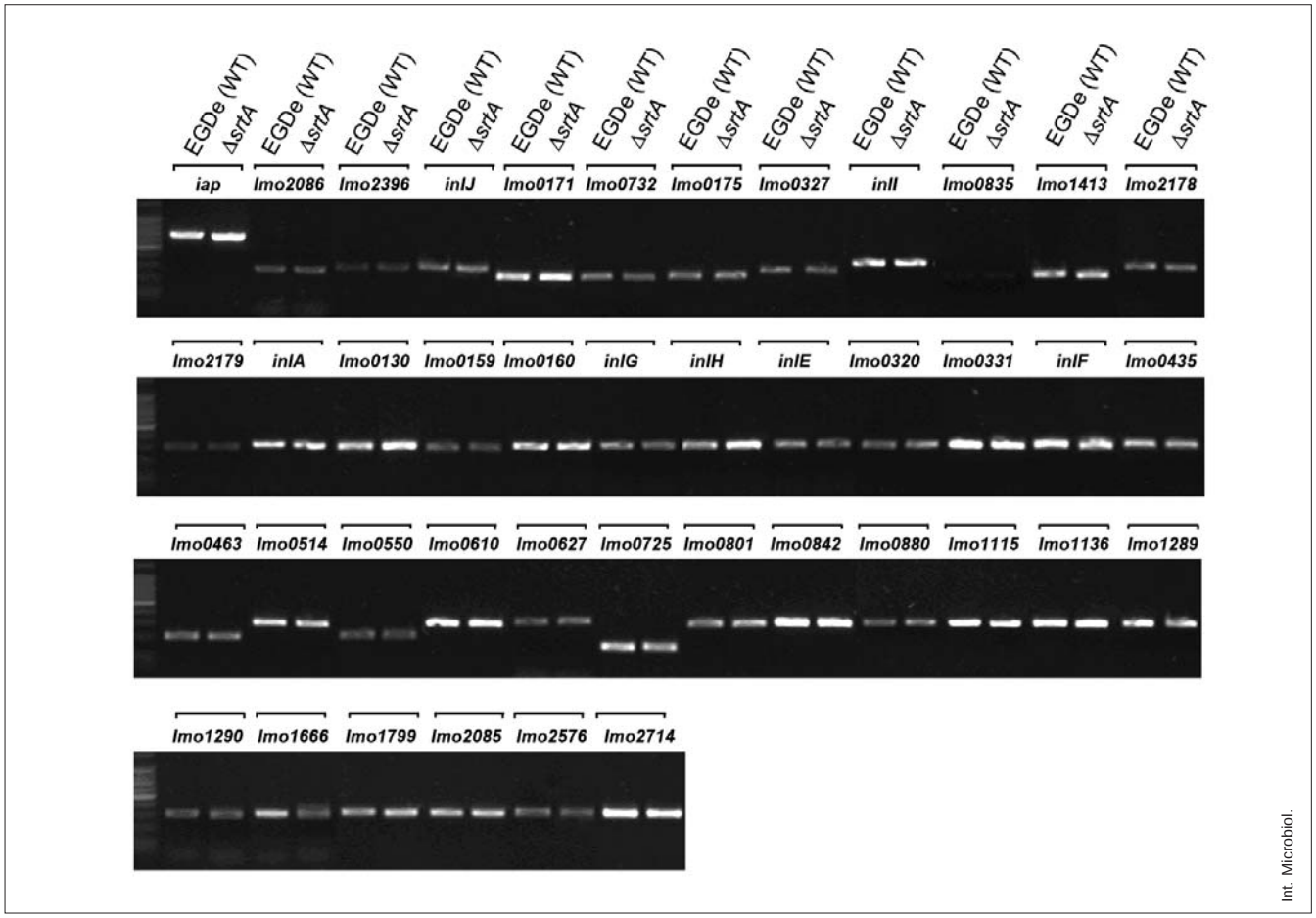

Fig. 1. As seen in real-time PCR (RT-PCR) assays, there was no difference between EGDe (wild-type) and BUG1777 (Δ*srtA*) isogenic strains with respect to the expression of the 41 *Listeria monocytogenes* genes encoding LPXTG surface proteins. Bacteria were grown to exponential phase (OD = 0.2) in BHI medium at 37 ºC. The *iap* gene, encoding the peptidoglycan hydrolase P60, which is not recognized by SrtA, was included as control. Note that, despite the variability in expression between some genes encoding LPXTG proteins, there were no differences among the two strains tested.

Among the LPXTG proteins better detected by our immune sera were Lmo0130, Lmo0159, Lmo0160, Lmo0263 (InlH), Lmo0610 and Lmo0880. Therefore, the relative amounts of these six LPXTG proteins and Lmo0434 (InlA) were examined in the cell-wall and extracellular-medium fractions of exponentially growing isogenic wild-type and Δ*srtA* strains. The cell-wall-associated protein Iap (also known as P60), which binds to peptidoglycan non-covalently by its LysM domain, and the SrtB substrate Lmo2185 (SvpA) were included as controls of surface protein not recognized by SrtA. In the Δ*srtA* strain, the lack of SrtA resulted in a significant decrease in LPXTG protein levels in the cell wall and a concomitant increase in the amount of protein released into the extracellular medium (Fig. 2A). As expected, the mis-sorting of the LPXTG proteins by the Δ*srtA* mutant was not observed for the P60 and SvpA proteins, which do not depend on SrtA for their cell-wall association (Fig. 2A). Instead, consistent with the mode of association reported for these two proteins, P60 was mostly secreted into the medium while SvpA was retained in the cell wall by the covalent linkage mediated by SrtB (Fig. 2A). Of interest, the levels of some the LPXTG proteins examined, such as Lmo0610 and Lmo0880, were lower in the Δ*srtA* mutant than in the wild-type strain, when considering both the cell-wall and the extracellular fractions (Fig. 2A). Slight decreases in the amounts of two other LPXTG proteins, Lmo0159 and Lmo0160, were also noted while the remaining two LPXTG proteins examined, Lmo0263 (InlH) and Lmo0434 (InlA), were detected in similar amounts irrespective of the presence/absence of SrtA (Fig. 2A).

Of note, some of these LPXTG proteins were detected in the Western immunoassays as double or multiple bands. However, they were found to be specific when compared

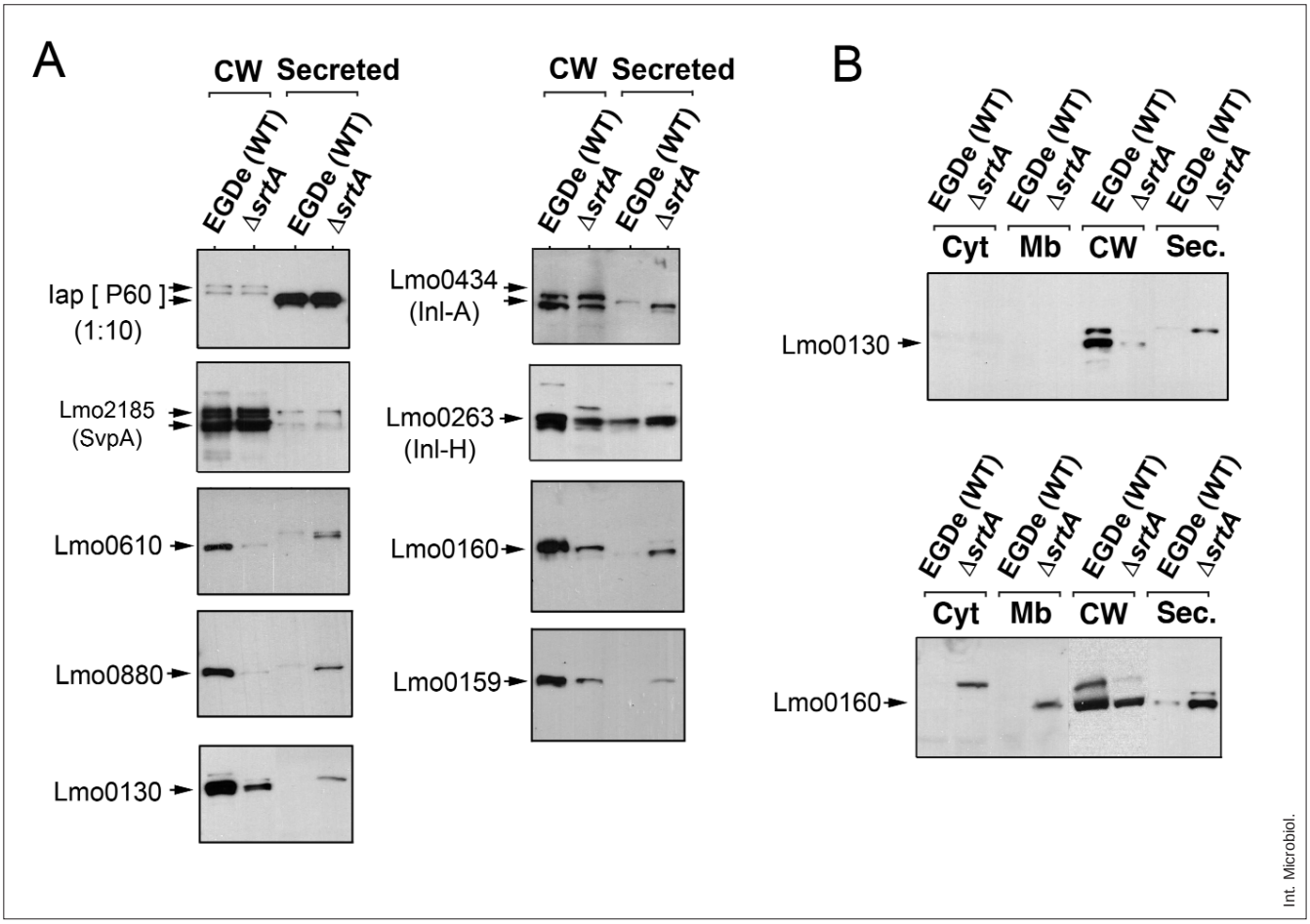

Fig. 2. (**A**) Western-blot assays showing the relative levels of distinct LPXTG proteins (Lmo0130, Lmo0159, Lmo0160, Lmo0263 -InlH-, Lmo0434 -InlA-, Lmo0610, and Lmo0880) in the cell wall (CW) and extracellular medium ('Secreted') fractions of *L. monocytogenes* EGDe (wild-type) and BUG1777 (Δ*srtA*) strains grown to exponential phase (OD_{600} = 0.2) in BHI medium at 37 ºC. Non-SrtA substrates, such as the peptidoglycan hydrolase Iap (P60) and the SrtB substrate Lmo2185 (SvpA), were run as controls. (**B**) Western assays showing the distribution of the LPXTG proteins Lmo0130 and Lmo0160 in subcellular fractions of bacteria grown as in panel A. Cyt: cytosol; Mb: membrane; Sec.: secreted.

with mutants defective in these LPXTG proteins (data not shown). This multiplicity of bands for sortase substrates in both peptidoglycan and secreted fractions has been previously reported [2] and might reflect the attachment of muropeptides to proteins extracted from the cell wall by muramidase digestion. In these two fractions (cell wall and secreted), differences in protein mobility in the gel are not likely to be due to unprocessed precursor forms of these proteins, as they would be almost certainly retained at the membrane.

To determine whether the LPXTG proteins detected at lower levels in the cell wall and extracellular fractions of the Δ*srtA* mutant were produced at normal levels but accumulated in the membrane or cytosol, these two subcellular fractions were examined for the representative cases of Lmo0130 and Lmo0160. As shown in Fig. 2B, Lm0130 was not detected in either the cytosol or the membrane fraction of wild-type and Δ*srtA* strains. These data suggested that Lmo0130 could be subjected to post-transcriptional regulation, with the involvement of SrtA. By contrast, Lmo0160 was detected in the cytosol and membrane fractions of the Δ*srtA* mutant (Fig. 2B). Considering that Lmo0160 was also detected in the cell-wall and extracellular medium fractions and that its total amount was indistinguishable between wild-type and Δ*srtA* strains (Fig. 2B), the production of this protein might have been constitutive. Nonetheless, it remains to be explained why a portion of Lmo0160 molecules remained bound to the peptidoglycan in the absence of SrtA. This behavior, shared by other LPXTG proteins such as Lmo0263 (InlH) and Lmo0434 (InlA) (Fig. 2A), suggests that the presence of additional modules of these proteins favored the retention of

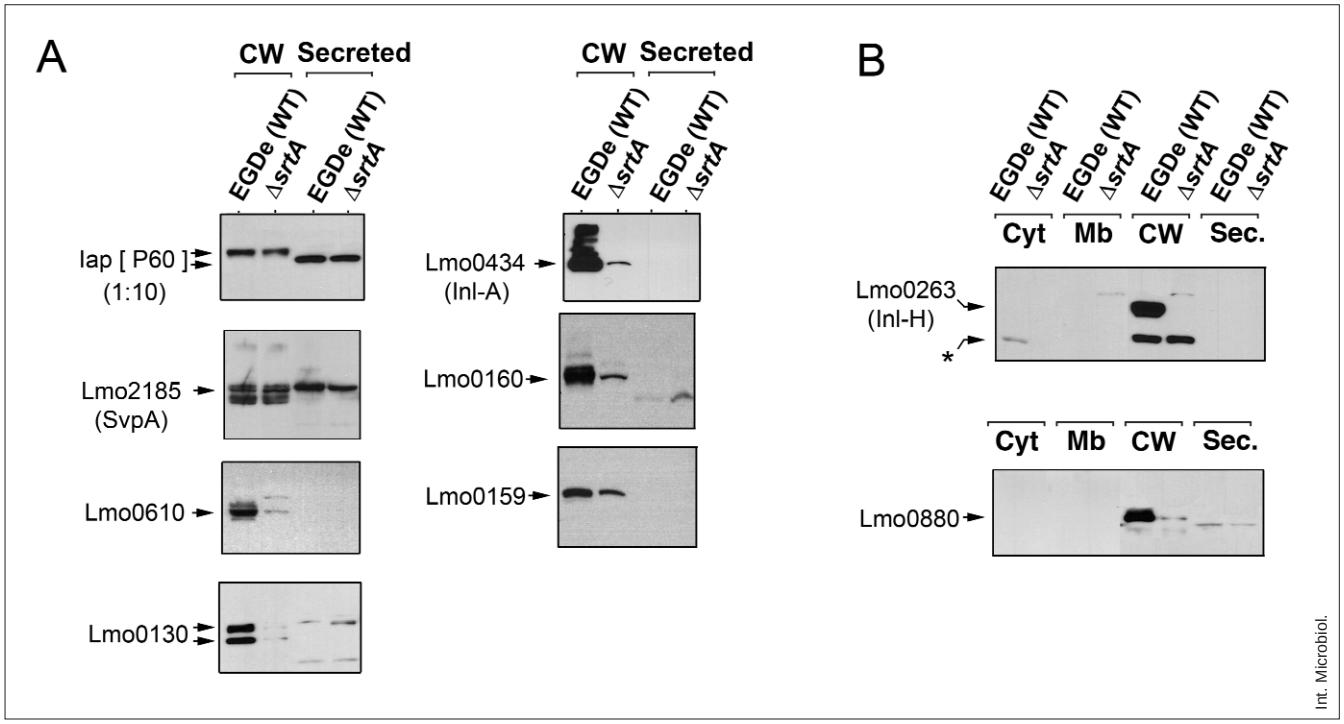

Fig. 3. (**A**) Western-blot assays depicting the relative levels of the LPXTG proteins Lmo0130, Lmo0159, Lmo0160, Lmo0434 -InlA-, and Lmo0610 in the cell wall (CW) and extracellular medium ('Secreted') fractions of *L. monocytogenes* EGDe (wild-type) and BUG1777 (Δ*srtA*) strains grown to stationary phase (OD_{600} = 1.0) in BHI medium at 37 ºC under non-shaking conditions. Non-SrtA substrates, such as the peptidoglycan hydrolase Iap (P60) and the SrtB substrate Lmo2185 (SvpA), were run as controls. (**B**) Western blots showing the distribution of the SigB-regulated LPXTG proteins Lmo0263 (InlH) and Lmo0880 in subcellular fractions prepared from wild-type and Δ*srtA* bacteria grown as in panel A. In the anti-InlH immunoblot, the asterisk denotes an unspecific band not related to the LPXTG protein. Cyt: cytosol; Mb: membrane; Sec.: secreted.

the protein in the cell wall. The existence of additional peptidoglycan-binding domains in LPXTG surface proteins is, to our knowledge, unreported. Note that *in-silico* analyses have demonstrated that the *L. monocytogenes* Lmo0880 harbors a LysM domain [1], as do other surface proteins not recognized by sortases, such as P60. Our subcellular fractionation studies showed, however, that Lmo0880 was not extensively retained in the Δ*srtA* mutant. This result is consistent with the low percentage of P60—a surface protein that includes two LysM domains—able to withstand the harsh peptidoglycan purification method, involving extensive boiling in SDS-containing solutions (Fig. 2A) [26]. Overall, our observations point to as-yet-unidentified modes of association—and thus independent of the action of SrtA—between peptidoglycan and certain LPXTG surface proteins. These associations would presumably also rely on domain(s) other than LysM and perhaps contained in LPXTG proteins, such as Lmo0263 (InlH), Lmo0434 (InlA) and Lmo0160. That some of these proteins might be able to form macromolecular complexes is also an interesting possibility.

Growth arrest also regulates the production of certain *L. monocytogenes* LPXTG proteins in an SrtA-dependent manner. Previous studies have shown that certain *L. monocytogenes* LPXTG proteins, namely, Lmo0263 (InlH), Lmo0610, Lmo0880, and Lmo2085, are regulated by the alternative sigma factor SigB [17,19,25]. SigB is also known to control cellular responses to diverse stresses, such as high osmolarity and acidic pH, to govern bacterial adaptation to conditions of nutrient limitation, and to modulate the expression of other virulence genes (reviewed in [22]). Our proteomic studies showed that many LPXTG proteins are up-regulated when *L. monocytogenes* is grown to stationary phase [8,13]. Based on these observations, we analyzed whether the up-regulation of these LPXTG proteins in growth-arrested cells requires a functional sortase enzyme. Thus, the distribution of different LPXTG proteins was examined in cell-wall and extracellular-medium extracts of *L. monocytogenes* wild-type and Δ*srtA* strains grown to stationary phase. In a first set of experiments, the distribution of Lmo0130, Lmo0159, Lmo0160, Lmo0610,

and Lmo0434 (InlA) was examined. In all cases, the relative amount of these LPXTG proteins anchored to the peptidoglycan was lower in the Δ*srtA* mutant (Fig. 3A).

The fact that most of these LPXTG proteins were not detected in the extracellular medium suggested that the lack of SrtA blocked their up-regulation during the transit of *L. monocytogenes* to stationary phase. Taken together, these data support a model in which the lack of SrtA affects the production of LPXTG proteins, at least those tested, when the cells reach stationary phase. This effect was especially evident for Lmo0434 (InlA), since during exponential phase it was detected at similar levels in wild-type and Δ*srtA* strains (see Fig. 2A). Control experiments with the SigB-regulated LPXTG proteins Lmo0263 (InlH) and Lmo0880 demonstrated that their marked decrease in the Δ*srtA* mutant grown to stationary phase was not due to an accumulation in the cytosol or the membrane (Fig. 3B). In addition, non-SrtA substrates, such as P60 and Lmo2185 (SvpA), were detected both in the cell wall and, especially, in the extracellular fractions (Fig. 3A). This finding excludes the possibility of a general degradation of LPXTG proteins in the extracellular medium by proteases secreted during stationary phase. Overall, our findings provide support for a novel mechanism of regulation of LPXTG surface proteins, in which SrtA seems to contribute mostly when the bacteria reach stationary phase. This mechanism in essence acts as a negative form of regulation of LPXTG proteins when StrA, which anchors these substrate proteins to cell-wall peptidoglycan, is functionally altered. Future studies will need to address the exact regulatory elements involved and the mechanism(s) underlying their regulatory activities in the distinct phases of bacterial growth.

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Competing interests. None declared.

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