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**A systematic proteomic analysis of *Listeria monocytogenes* house-keeping protein secretion systems**

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## SUMMARY

*Listeria monocytogenes* is a firmicute bacterium causing serious infections in humans upon consumption of contaminated food. Most of its virulence factors are secretory proteins either released to the medium or attached to the bacterial surface. *L. monocytogenes* encodes at least six different protein secretion pathways. While great efforts have been made in the past to predict secretory proteins and their secretion routes using bioinformatics, experimental evidence is lacking for most secretion systems. Therefore, we constructed mutants in the main housekeeping protein secretion systems, which are the Sec-dependent transport, the YidC membrane insertases SpoIIIJ and YqjG, as well as the twin-arginine pathway, and analysed their secretion and virulence defects. Our results demonstrate that Sec-dependent secretion and membrane insertion of proteins via YidC proteins are essential for viability of *L. monocytogenes*. Depletion of SecA or YidC activity severely affected protein secretion, whereas loss of the Tat-pathway was without any effect on secretion, viability and virulence. Two-dimensional gel electrophoresis combined with protein identification by mass spectrometry revealed that secretion of many virulence factors and of enzymes synthesizing and degrading the cell wall depends on the SecA route. This finding was confirmed by SecA inhibition experiments using sodium azide. Analysis of secretion of substrates typically dependent on the accessory SecA2 ATPase in wild type and azide resistant mutants of *L. monocytogenes* revealed for the first time that SecA2-dependent protein secretion also requires the ATPase activity of the house-keeping SecA protein.

## INTRODUCTION

*Listeria monocytogenes* is a facultative pathogenic firmicute bacterium that is found frequently in nature where it lives as a saprophyte in the soil and on decaying plant material. Due to its ubiquitousness, it frequently enters the food chain giving rise to listeriosis outbreaks that often reveal a high rate of fatal cases in humans (1, 2). Listeriosis typically establishes as a self-limiting febrile gastroenteritis in otherwise healthy individuals. However, it can turn into a more severe condition especially in elderly or immune-compromised patients as well as in pregnant women, where it can lead to meningitis, encephalitis, septicaemia, abortions and neonatal infections (2-4). Upon entry into the host due to ingestion of contaminated food, the bacterium activates expression of virulence genes that are under the control of the transcriptional activator PrfA (5). These virulence factors enable *L. monocytogenes* (i) to bind to host cell surfaces in order to induce its uptake into host cells in a phagocytosis-like process (internalins), (ii) to break down the phagosomal membrane for escape into the host cell cytoplasm (listeriolysin, metallo-protease, and phospholipases), and (iii) to move inside the cytosol of infected cells and to finally spread to uninfected neighbour cells (the actin remodelling protein ActA and phospholipases) (1, 6, 7). Like most virulence factors from other bacterial pathogens, these proteins are translocated across the cytoplasmic membrane in order to be presented on the cell surface or released into the extracellular milieu (8). This underpins the importance of protein secretion pathways as determinants for the correct subcellular targeting of virulence factors and thus for pathogenicity of *L. monocytogenes* and other bacterial pathogens. Due to this reason, components of protein translocating systems have attracted attention as potential drug targets (9, 10).

The presence of six different protein secretion pathways has been predicted in *L. monocytogenes* based on bioinformatic analyses (8) (Tab. 1). This includes the general secretion (Sec) system which is composed of the protein conducting SecYEG channel and the

auxiliary SecDF and YajC proteins. Protein secretion is driven by the SecA ATPase, which energizes translocation of secreted and trans-membrane proteins through the membrane-embedded SecYEG pore (11, 12). SecA binds to N-terminally located signal sequences in pre-proteins (13) and guides them to SecYEG (11, 14). Repeated cycles of ATP hydrolysis drive large conformational changes in SecA pushing the pre-protein through the SecYEG channel (11, 15, 16). The signal peptide is later cleaved off (14), and the mature protein is either released into the extracellular space or linked to the bacterial surface (17-19). The secretion of some listerial proteins requires the presence of the accessory SecA2 ATPase (20). This includes the autolysins p60 (CwhA) and MurA (NamA), both containing Sec-type signal sequences (17, 21), among a few other proteins (20, 22-24). SecA2 proteins are found in many Gram-positive bacteria in addition to their housekeeping SecA homologues. *L. monocytogenes* SecA2 has all key domains characteristically found in SecA proteins and shares 44% identity (62% similarity) with listerial SecA (25). SecA2 proteins typically serve the secretion of a limited number of proteins that often are linked to virulence (26). However, presently it is not clear why exactly these substrates require SecA2 for their translocation (26).

For membrane insertion, transmembrane segments of integral membrane proteins are laterally released from the SecYEG pore in a process involving the YidC membrane insertases (27). For some substrates, however, YidC proteins can even act as Sec-independent membrane insertases (28). Firmicute bacteria such as *Bacillus subtilis* or *L. monocytogenes* contain two YidC homologues, SpoIIIJ and YqjG (27, 29). Both proteins have distinct as well as overlapping substrate spectra, at least in *B. subtilis* (30, 31). Integral membrane proteins are translocated co-translationally. As soon as their nascent chain exits the ribosome, they are recognized by the signal recognition particle (SRP), a ribonucleoprotein complex composed of the GTPase Ffh, its newly identified modulator YlxM (32) and the SRP 4.5S RNA. The SRP guides the ribosome-nascent chain complexes to the SecA-SecYEG translocon via the transmembrane SRP receptor FtsY (33, 34). While Sec substrates are translocated in the

unfolded state, substrates of the twin-arginine transport (Tat) are secreted as folded proteins that even can be complexed with co-factors (35). The *L. monocytogenes* Tat system is composed of the polytopic transmembrane protein TatC required for substrate recognition and the small membrane spanning TatA protein (8), forming the pore through which protein transport is driven by proton motive force (35). Next to these systems, four additional, but more specialized protein secretion pathways are present in the *L. monocytogenes* genome: The flagellum exporter for translocation of flagellar proteins, the fimbriae exporter for secretion of pseudo-pilus subunits, which contribute to phagosomal escape (36), two holins for secretion of phage endolysins, as well as the WXG100 system supposed to mediate secretion of short, ~100 amino acids long substrate proteins with the typical WXG motif (Tab. 1) (8).

The contribution of most *L. monocytogenes* secretion systems to general protein secretion has not been studied experimentally. Whereas SecA2-dependent protein secretion significantly contributes to *L. monocytogenes* virulence (20, 37), the Tat as well as the WXG100 system seemed to be dispensable for listerial pathogenicity (38, 39). However, a recent investigation of *L. monocytogenes* SecDF showed that the Sec system contributes to virulence factors secretion (40). According to *in silico* predictions using the presence of signal peptides, trans-membrane domains and surface retention signals as typical characteristics of secreted proteins, roughly one third of all 2,853 *L. monocytogenes* proteins (41) is either inserted into the cytoplasmic membrane (686), or translocated across the membrane to be released into the extracellular space (80) or to be presented on the surface of the cell (143) (17). This approach has also facilitated an assignment of all secreted proteins to the different protein secretion mechanisms (17). However, the only secretion system for which these predictions have been validated by experimental data is the SecA2-dependent translocation route.

Here, we studied the different protein secretion systems of *L. monocytogenes*. Mutant strains lacking or conditionally expressing the SecA ATPase, the YidC membrane insertases

SpoIIIJ and YqjG, as well as the Tat system components TatAC were characterized with regard to *in vitro* and *in vivo* growth and possible secretion defects. Mutant strains devoid of the *fliI* (flagellar export), *yukA* (WXG100) and *spoIIIAH* genes (encoding a ring-forming membrane embedded protein possibly involved in protein secretion, see below) were also analyzed. While the three latter mutants did not show any phenotypic differences compared to wild type, SecA and the YidC proteins were clearly required for viability and contribute to bulk protein secretion under standard laboratory conditions. Proteomic analyses of secretion patterns and construction of epitope tagged substrate proteins for analysis of their secretion by Western blotting enabled us to assign secretion substrates of the SecA-, the YidC- and the TatAC-dependent protein secretion routes.

## EXPERIMENTAL PROCEDURES

### Bacterial strains and growth conditions

Table 2 lists all bacterial strains used in this study. Cells of *L. monocytogenes* were routinely cultivated on brain heart infusion (BHI) agar or in BHI broth. Where necessary, antibiotics and supplements were added at the following concentrations: erythromycin (5 µg/ml), kanamycin (50 µg/ml), X-Gal (100 µg/ml) and IPTG (0.005-1 mM). For all cloning procedures *Escherichia coli* TOP10 was used as the standard plasmid host (42).

### General methods, DNA manipulation and oligonucleotides

Transformation of *E. coli* and *L. monocytogenes* as well as extraction of plasmid DNA were performed according to established protocols (42, 43). Enzymatic manipulations of DNA were performed as described in the manufacturer's guidelines. All oligonucleotides are listed in Table 3.

### Construction of plasmids and strains

Plasmid pSH183 was constructed to delete the *spoIIIAH* gene from the chromosome of *L. monocytogenes*. For this purpose, up- and downstream fragments of *spoIIIAH* were amplified in a PCR using the oligonucleotides SHW1/SHW2 and SHW3/SHW4, respectively. Both fragments were joined together by ligation after their ends had been made compatible by Sall restriction. The joined fragment was amplified from the ligation mixture using SHW1/SHW4 and the resulting PCR product was cloned into pMAD using NcoI/Sall.

Plasmid pSH184, designed for the deletion of *yukA*, was constructed analogously. *yukA* up- and downstream fragments were generated by PCR with SHW21/SHW22 and SHW23/SHW24, respectively. Both fragments were cut with NotI and fused together by

ligation. The desired fusion product was amplified in a second PCR with the primer pair SHW21/SHW24 and cloned into pMAD using EcoRI/SalI.

Plasmid pSH189 allows deletion of the *yqjG* gene and was obtained by removal of the *yqjG* ORF from plasmid pSH188 using primers SHW46/SHW47. The resulting PCR product was SalI cut and circularized in a ligation reaction. Plasmid pSH188 had been obtained by cloning of the entire *yqjG* region into pMAD using primers SHW45/SHW48 and BamHI/NcoI as the restriction enzymes.

To allow for conditional expression of *yqjG*, we constructed plasmid pSH208. This plasmid was obtained by subcloning of the BamHI/SalI *yqjG* fragment from plasmid pSH199 into pIMK3 cut with the same enzymes. Plasmid pSH199 had been constructed by cloning of the *yqjG* gene into pIMK2 using the oligonucleotides SHW99/SHW100 and BamHI/SalI digestion.

Plasmid pSH341 was generated in order to construct a *spoIIJ* mutant. Since cloning difficulties forced us to exclude a functional copy of the upstream *rnpA* gene from cloning, the strategy for generation of a *spoIIJ* mutant was slightly changed. A fragment which starts from inside the *rnpA* gene and reaches to the 345<sup>th</sup> nucleotide of *spoIIJ* and a fragment just downstream of *spoIIJ* were amplified using the oligos SHW388/SHW390 and SHW7/SHW8, respectively (SHW390 introduced a premature stop codon into *spoIIJ* after its 115<sup>th</sup> amino acid). Both fragments were cut SalI and fused together by ligation. The desired fusion fragment was then amplified in a second PCR using the oligonucleotides SHW388/SHW8 and cloned into pMAD using BamHI/NcoI.

For IPTG-inducible expression of *secA*, plasmid pSH352 was constructed. This plasmid was obtained by cloning the *secA* open reading frame, which was amplified from genomic DNA in a PCR using the primers SHW419/SHW420, into plasmid pIMK3 after NcoI/SalI restriction. The *azi-1* mutation (*secA128A*) was brought into pSH352 in a quickchange PCR using SHW490/SHW491 as the mutagenic primers. This yielded plasmid

pSH383. The *tatAC* deletion plasmid pSH360 was constructed in several steps. First of all, a 2.7 kb fragment comprising the *tatAC* operon as well as surrounding genomic regions was amplified using the primer pair SHW411/SHW412 and blunt-end cloned into SmaI cut pUC19, resulting in plasmid pSH351. The *tatAC* operon was then deleted from this plasmid in a PCR using the primers SHW413/SHW414 (=pSH353). Finally, the NcoI/SalI  $\Delta$ *tatAC* fragment of pSH353 was then subcloned into pMAD cut with the same enzymes.

Plasmid pSH361, designed for deletion of *secA*, was constructed analogously. A 4.1 kb fragment comprising the *secA* open reading frame as well as surrounding regions was amplified using the oligonucleotides SHW415/SHW416 and the resulting fragment was blunt-end cloned into SmaI cut pUC19 (=pSH356). The *secA* gene was then removed from pSH356 in a PCR using the primer pair SHW417/SHW418, resulting in plasmid pSH357. Finally, the NcoI/SalI  $\Delta$ *secA* fragment of pSH357 was subcloned into pMAD which had been digested with the same enzymes.

For immunogenic detection of Lmo2201 and Lmo0367, we first constructed plasmids pSH366 and pSH367, respectively. Both plasmids were obtained by amplification of *lmo2201-strep* and *lmo0367-strep* fragments from chromosomal DNA using SHW441/SHW442 and SHW439/SHW440, respectively, and subsequent cloning of both fragments into SmaI cut pAUL-A (the reverse primers introduced a C-terminal Strep-tag). Plasmids pSH366 and pSH367 were then inserted at the *lmo2201* (strain LMS105) and *lmo0367* loci (LMS107) of *L. monocytogenes* EGD-e in order to obtain full-length proteins fused to C-terminal Strep-tags. *lmo2201-strep* and *lmo0367-strep* were amplified from genomic DNA of strains LMS105 and LMS107, respectively, using the primer pairs SHW463/464 and SHW465/SHW116. The resulting fragments were cut NcoI/PstI and ligated to pIMK3 cut with the same enzymes. This yielded plasmid pSH376 for inducible expression of Lmo2201-Strep and plasmid pSH377 for inducible Lmo0367-Strep expression. In order to enhance the immunogenicity of Lmo0367-Strep further, a second Strep-epitope was

introduced into the protein replacing the VDNDMLPP loop at position 124 to 131. This was obtained by quickchange mutagenesis of plasmid pSH377 using the primers SHW474/SHW475 and yielded plasmid pSH380.

In order to facilitate immunogenic detection of InlA, plasmid pSH386 was constructed. Primers SHW497/SHW498 were used to amplify an *inlA* fragment comprising the amino acid codons 490-759 in a way that the LPXTG motif was replaced by a C-terminal FLAG-tag. The resulting fragment was blunt end cloned into SmaI cut pAUL-A. Plasmid pSH386 was then inserted into the *inlA* loci of strains EGD-e, LMS102, and LMS129, and plasmid integration was verified by PCR.

Plasmids designed to integrate at the *attB* site of the tRNA<sup>Arg</sup> locus (pSH208, pSH352, pSH376, pSH377, pSH380, pSH383) were transformed to *L. monocytogenes* EGD-e and kanamycin resistant clones were isolated. Plasmid integration was verified by PCR. Gene deletions using derivatives of the pMAD vector were carried out according to a previously described protocol (44). All deletions were confirmed by PCR.

### **Protein purification**

His<sub>6</sub>-SecA was overexpressed from the expression plasmid pET19b-*secA* in *E. coli* BL21. Expression was induced by the addition of 1 mM IPTG to exponentially growing cultures, and the proteins were purified using Ni-NTA sepharose (Amersham). After purification, protein containing fractions were pooled, dialysed against a buffer containing 50 mM Tris-HCl pH 7.5, 200 mM NaCl and 1 mM DTT, and aliquots were stored at -80°C.

### **Isolation of protein fractions for SDS-PAGE, Western blotting and zymography**

*L. monocytogenes* strains were grown in BHI broth to an optical density of OD<sub>600</sub> = 1.0 and harvested by centrifugation. The cell pellet was washed once in ZAP buffer (10 mM Tris-HCl pH7.5, 200 mM NaCl) and resuspended in ZAP buffer containing 1 mM PMSF. Cells were

disrupted by sonification and cell debris was removed by centrifugation. The resulting supernatant was considered to contain the total cellular protein fraction. In order to separate membrane proteins from the soluble cytosolic proteins, this fraction was ultracentrifuged at 100.000 x g for 30 min at 4°C. The resulting supernatant contained the soluble cytoplasmic proteins and the pellet corresponding to the membrane fraction was resuspended in 100 µl ZAP buffer. Extracellular proteins were routinely isolated by precipitation from the cell-free medium using trichloroacetic acid (10% w/v final concentration) and over-night incubation at 4°C. Precipitated proteins were collected by centrifugation at 6000 x g for 1 h at 4°C, dried and resuspended in 8 M urea.

Protein samples were separated by standard SDS polyacrylamide electrophoresis and coomassie-stained or transferred onto positively charged polyvinylidene fluoride (PVDF) membranes employing a semi-dry transfer unit. Immune-staining was carried out using polyclonal rabbit antisera recognizing SecA (45), p60 (MyBioSource, USA), ActA (46), PlcA, PlcB (47), or monoclonal mouse antisera raised against the FLAG-epitope (Sigma) or the Strep-tag (IBA) as the primary, and anti-rabbit or anti-mouse immunoglobuline G conjugated to horseradish peroxidase as the secondary antibody. The peroxidase conjugates were visualized on the membrane by the ECL chemiluminescence detection system (Thermo Scientific).

In order to detect MurA, secretome samples were separated on 12% SDS-PAGE gels containing 0.2% (w/v) autoclaved and lyophilized *M. lysodeikticus* ATCC 4698 cells. Cell walls were degraded during a 24 hour incubation step during which the gel was kept in 25 mM Tris-HCl pH7.5 + 0.1% (v/v) Triton X-100 at room temperature. Autolysin activity was detected as brightened zones of peptidoglycan hydrolysis. When photographed in front of a dark background, these appear as black bands.

### **Depletion conditions and isolation of extracellular proteins for 2D-PAGE**

For the identification of SecA-dependent extracellular proteins, it was vital to identify growth conditions allowing optimal depletion of SecA without unnecessarily impairing growth rate of the SecA depletion strain LMS104. Pilot experiments showed that this could not be achieved by simply adjusting the IPTG concentration at the preparative scale needed for proteome analysis. Therefore, SecA depletion was conducted in the following way: Strain LMS104 was grown overnight in BHI broth containing 1 mM IPTG. These cells were used to start 500 ml BHI cultures containing 100  $\mu$ M IPTG (SecA expression) or no IPTG (SecA depletion) at an initial optical density of 0.05 and cultivated at 37°C until the cells had reached an optical density of 0.2. In order to remove proteins which were secreted while SecA depletion was still incomplete, the growth medium was replaced with fresh pre-warmed BHI broth that again either contained 100  $\mu$ M IPTG or no IPTG. Cultivation was continued until an OD<sub>600</sub> of 1.5. Under such depletion conditions, LMS104 grew with almost similar growth rates in the absence of IPTG as compared to growth in the presence of the inducer. For depletion of YqjG in strain LMS101, LMS101 cells were grown over night in BHI broth containing 1 mM IPTG and cells from this culture were used to start a depletion culture (no IPTG added) the next morning. After cultivation for 24 hours in the absence of IPTG, YqjG-depleted LMS101 cells were used to inoculate cultures either containing 100  $\mu$ M IPTG (YqjG expression) or 5  $\mu$ M IPTG (YqjG depletion). Cultivation was continued until the culture supplemented with 100  $\mu$ M IPTG reached an OD<sub>600</sub> of 1.5. Cells were removed from culture supernatants by centrifugation. The resulting supernatants were centrifuged a second time in order to remove residual cells and mixed with trichloroacetic acid afterwards at a final concentration of 10% (w/v). Proteins were precipitated over night at 4°C and collected by centrifugation for 1 h at 6000 x g and 4°C. Protein pellets were then washed once in 96% ethanol, four times in 1 ml of ice-cold acetone, and then left to air-dry. Protein re-solubilisation was done in an aqueous solution containing 8 M urea 2 M thiourea for 30 min at room temperature and insoluble

material was removed by centrifugation. Protein concentration was determined using the Roti® Nanoquant reagent.

### **Two-dimensional polyacrylamid gel electrophoresis (2D-PAGE)**

2D-PAGE was performed using the immobilized pH gradient (IPG) technique as described previously (48). In the first dimension, 100 µg of extracellular protein extracts were separated on IPG strips (GE-Healthcare, Uppsala, Sweden) covering a linear pI range of 3 to 10. In the second dimension, proteins were separated according to their molecular weight using SDS-PAGE as described previously (49). For protein detection, the obtained 2D gels were fixed with 40% (v/v) ethanol and 10% (v/v) acetic acid for one hour, washed with distilled water, stained overnight with the fluorescent dye Krypton<sup>TM</sup> according to manufacturer's instructions (Thermo Scientific, Waltham, MA, USA) and then scanned using a Typhoon 9400 Variable Mode Imager (GE Healthcare, Little Chalfont, UK).

### **Protein identification**

Protein spots of interest were excised automatically from the gel using an Ettan spot picker (GE Healthcare, Little Chalfont, UK) and transferred into a 96-well microtiter plate. In-gel digestion with sequencing grade trypsin (Promega, Madison, WI), and extraction of peptides was carried out using the Ettan Spot Handling Workstation (GE Healthcare, Little Chalfont, UK) according to the protocol described by Eymann *et al.* (50).

Identification of proteins by Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) and MALDI-TOF-TOF MS was carried out as published previously (51). MALDI-TOF measurement was performed on the AB SCIEX TOF/TOF<sup>TM</sup> 5800 Analyzer (AB Sciex/MDS Analytical Technologies). Spectra were recorded in reflector mode (mass range: 900-3700 Da). When the autolytic fragment of trypsin with the mono-isotopic (M+H)<sup>+</sup> m/z at 2211,104 reached the signal to noise ratio (S/N) of at least 40, an internal calibration

was performed as one-point-calibration using this peak. The standard mass deviation was less than 0.15 Da. When the automatic mode failed, the calibration was carried out manually.

The five most intense peaks from the TOF-spectra were selected for MS/MS analysis. For one main spectrum 20 sub-spectra were accumulated using a random search pattern. The internal calibration was performed as one-point-calibration, when the mono-isotopic arginine (M+H)<sup>+</sup> m/z at 175.119 or lysine (M+H)<sup>+</sup> m/z at 147.107 reached the signal to noise ratio of at least 5. Peak lists were created using the GPS Explorer<sup>TM</sup> software version 3.6 (build332). For database search the mascot search engine version 2.4.0 (Matrix Science Ltd, London, UK) and the *L. monocytogenes* EGD-e sequence (extracted from NCBI at the 30<sup>th</sup> October 2012) was used. Searches were restricted to the genus *Listeria* (249420 entries) and search parameters were set in the following way: Allowed missed cleavage: 1; fixed modifications: no; variable modifications: carbamidomethylation and oxidation [M]; mass tolerance for precursor ions: 100 ppm; mass tolerance for fragment ions: 0.55 Da. Protein scores greater than 66 are significant (p<0.05). The cut-off ion score/expectation value for accepting individual MS/MS spectra was 16. Peptide mixtures that yielded a mowse score of at least 66 were regarded as positive identifications. For a definite identification, each protein had to be identified at the same position on 2D gels of two biologically independent samples.

### **Protein quantification and statistical analyses**

For quantitation of cytoplasmic proteins, the 2D gel image analysis was performed using Delta2D v4.3 (Decodon, Greifswald, Germany). Gel to gel variations of spot positions on 2D gel images were compensated by warping of all images. Gel images generated from three biological replicates were used to create a virtual fusion gel which was used for spot detection and spot mask editing. Where necessary, automatically calculated spot borders were corrected and optimized manually. The final spot mask was transferred from the fusion gel to each gel image of the project to ensure complete spot matching. The spot volumes of all protein spots

were normalized using total normalization and for each protein spot, ratios of signal intensities were calculated. For statistical analyses standardized data were loaded into TIGR multiexperiment viewer 4.4.1. To test whether the signal intensity of a given protein spot changed significantly, the t-test analysis based on permutation option, which is included in the Decodon software, was used. Only spots whose signal intensity changed at least two-fold ( $P < 0.01$ ) were considered as differentially secreted proteins.

### **Infection experiments**

Intracellular growth kinetics of listerial strains in HeLa cells were performed as described earlier (37). Briefly,  $10^5$  HeLa cells were seeded into a 24 multi well plate and grown in 1 ml high glucose DMEM medium (4.5 g/l glucose, 110 mg/l sodium pyruvate, 584 mg/l L-glutamine) supplemented with 10% fetal calf serum (FCS) at 37°C in a 5% carbon dioxide atmosphere one day before the infection experiment. An aliquot corresponding to  $2.5 \cdot 10^6$  bacterial cells was resuspended in 1 ml of fresh DMEM without FCS and used to infect the HeLa cell monolayer during an incubation step of 1 h at 37°C. The wells were washed three times with PBS and all extracellular bacteria were killed during another 1 h incubation step in DMEM (without FCS) containing 40 µg/ml gentamicin. After three more PBS wash steps the wells were covered with fresh DMEM (without FCS) containing 10 µg/ml gentamicin. Sampling was performed by lysing the cells in 1 ml of ice-cold PBS containing 0.1% Triton X-100. Serial dilutions were plated on BHI agar plates in order to count the recovered bacterial colonies.

For intracellular growth in macrophages,  $3 \times 10^5$  J774.A1 mouse ascites macrophages (ATCC) were seeded into the wells of a 24 multi well plate and cultivated as described above for one day at 37°C in a 5% CO<sub>2</sub> atmosphere. *L. monocytogenes* strains were grown overnight in BHI broth at 37°C and adjusted to an OD<sub>600</sub> of 0.2 which was further diluted (1:100) in DMEM not containing FCS. 50 µl aliquot of this resuspension ( $\sim 5 \times 10^4$  bacterial cells)

were used to infect the J774 cells. Infection and sampling was performed similarly as described above. But due to reduced adherence of J774 cells the number of PBS washes after infection and gentamicin treatment was reduced to one.

Cell-to-cell spread was analysed in a plaque formation assay using 3T3 L1 mouse embryo fibroblast-like cells according to previously published protocols (37, 52). 3T3 L1 cells were cultivated in 6 well plates and grown to confluency in DMEM medium containing 10% FCS for 2 days at 37°C in a 5% CO<sub>2</sub> atmosphere. Cell layers were washed three times with PBS and overlaid with fresh DMEM medium not containing FCS. 1 µl of a suspension of logarithmically growing bacteria which were diluted in BHI to an optical density of 0.2 was added to the cell culture and infection was allowed to proceed during an incubation step for 1 h at 37°C. Extracellular bacteria were washed off afterwards and the 3T3 L1 cells were overlaid with an agarose layer (which was prepared from two parts DMEM and one part 1.5% low melting point agarose in 0.9% NaCl, and also contained 10 µg/ml gentamicin). After 3 days of incubation, plaques were visualized by staining with a second layer of DMEM agarose which additionally contained 0.01% neutral red and 5 mM HCl.

### **Insect infection model**

*Galleria mellonella* larvae, purchased from fauna topics (Marbach, Germany), were reared at 32°C in darkness and on an artificial diet (22% maize meal, 22% wheat germ, 11% dry yeast, 17.5% bee wax, 11% honey and 11% glycerine) prior to use. Last instar larvae, each weighing between 250 and 350 mg, were used in all experiments as described previously (53).

In all experiments, fresh cultures of bacteria, prepared from an overnight culture, were used. Briefly, bacteria were grown in BHI broth at 37°C, harvested in the exponential growth phase and washed twice with 1 × PBS. The pellet was resuspended in 1 × PBS and the bacterial concentration was calibrated by optical absorption. Further dilutions were prepared in 1 × PBS to obtain required numbers of bacteria for infection. The infection of *G. mellonella*

was performed as previously described by Mukkerjee *et al.* (53). Briefly, the *L. monocytogenes* wild type strain EGD-e and its isogenic deletion mutants were separately injected ( $10^6$  CFU/larva) into the hemocoel of the last instar larvae and the infection was monitored at 37°C for seven days.

## RESULTS

### Deletion of *L. monocytogenes* secretion genes

All secretion mutants were constructed in the background of the *L. monocytogenes* reference strain EGD-e. This selection included a mutant allowing for conditional expression of the *secA* (*lmo2510*) gene encoding the housekeeping secretion ATPase SecA (strain LMS104). This strain was obtained by deletion of *secA* in a strain which contains an IPTG controlled copy of *secA* inserted at the *attB* locus since *secA* is an essential gene (43, 54). We then constructed mutants in the *spoIIIJ* (*lmo2854*, LMS100) and *yqjG* (*lmo1379*, LMS6) genes, both homologous to the *Escherichia coli* YidC membrane insertase required for membrane insertion of membrane-integral proteins. Since studies on the *yidC* homologs of *B. subtilis* demonstrated synthetic lethality of a *spoIIIJ yqjG* double deletion (30), a strain with deletions in both genes but containing an IPTG inducible copy of *yqjG* at the ectopic *attB* site was also generated (LMS101). Mutants with marker-less deletions of the *tatAC* operon (*lmo0362-lmo0361*, LMS103), encoding the components of the twin-arginine translocase, the *yukA* gene (*lmo0061*, LMS4) coding for the ATPase of the WXG100 secretion system, as well as the *lmo0791* gene (LMS5) which encodes a distant homologue of the *B. subtilis* SpoIIIAH protein were constructed, too. *B. subtilis* SpoIIIAH assembles into a dodecameric membrane-embedded ring (55), which is supposed to serve as a nutrient and signal conducting channel between the mother cell and the prespore in sporulating *B. subtilis* cells, and also shares similarity with the type III secretion inner membrane ring component EscJ from enteropathogenic *E. coli* (56). Furthermore, a previously constructed mutant lacking the *fliI* (*lmo0716*, LMS3) (37) gene was included in this study since we hypothesized that the flagellum export apparatus might serve the secretion of non-flagellum proteins as exemplified by the Cia proteins of *Campylobacter jejuni* (57).

### **Growth of *L. monocytogenes* secretion mutants**

All mutant strains were cultivated in BHI broth at 37°C and optical density was measured in hourly intervals until onset of stationary phase. No effects on growth rate, duration of lag phase or final optical density were observed for any of the deletion mutants in *spoIIIJ*, *yqjG*, *tatAC* (Fig. 1A-B), *yukA*, *fliI*, or in *spoIIIAH* (data not shown). In order to test the essentiality of the *secA* gene and the synthetic lethality of a *spoIIIJ yqjG* double knock-out, strains LMS104 ( $\Delta secA$  *attB::P<sub>help</sub>-lacO-secA lacI*) and LMS101 ( $\Delta spoIIIJ \Delta yqjG$  *attB::P<sub>help</sub>-lacO-yqjG lacI*) were grown overnight in BHI broth supplemented with 1 mM IPTG. Cells from these pre-cultures were used to inoculate cultures either containing or not containing 1 mM IPTG, but growth of both strains turned out to be still IPTG-independent at this stage of depletion. We hence continued to grow both strains in the absence of IPTG overnight and used these cells, which now were expected to be fully depleted for SecA and YqjG, as inocula for new cultures. As expected, both strains did not grow in BHI medium lacking IPTG. However, they grew as fast as the wild type strain in the presence of the inducer (Fig. 1C-D). In order to monitor expression of SecA during depletion, SecA was depleted from strain LMS104 as mentioned above and SecA-depleted LMS104 cells were used for cultures grown in the presence of increasing IPTG concentrations. Western blot analysis, using a polyclonal antiserum raised against *B. subtilis* SecA (45), revealed that SecA levels were minimal at 5  $\mu$ M IPTG but increased with increasing IPTG concentrations (Fig. 1E). Cross-reactivity of this antiserum against *L. monocytogenes* SecA is explained by the high degree of similarity that is shared between the SecA proteins of both organisms (68%). Taken together, these experiments confirmed the essentiality of the SecA secretion ATPase and the YidC membrane insertases for viability of *L. monocytogenes*.

### **Virulence of *L. monocytogenes* secretion mutants**

All mutant strains that did not require the presence of IPTG for viability were analyzed in a plaque formation assay using 3T3 mouse embryo fibroblasts. None of these mutants, however, did show a decrease in the number of plaques formed or in plaque size suggesting that invasion and cell-to-cell spread in these cells was not affected upon deletion of the *spoIIIJ*, *yqjG*, *tatAC* (Fig. 2A), *yukA*, *fliI* or *spoIIIAH* genes (data not shown). The pathogenic potential of mutants lacking the *spoIIIJ*, *yqjG*, or the *tatAC* genes was then studied in more detail. First we made use of a HeLa cell infection assay, which simultaneously monitors host cell invasion and intracellular multiplication rates. When compared to the wild-type strain no significant reduction in invasion rate was observed for any of these mutants. Also, the kinetics of intracellular multiplication of all three mutant strains inside HeLa cells were indifferent from wild type strain EGD-e (Fig. 2B). In order to measure intracellular multiplication in macrophages, the J774 mouse ascites macrophage line was used. This assay also did not reveal any indication for an attenuation of the mutants lacking *spoIIIJ*, *yqjG*, or *tatAC* (Fig. 2C).

As a next step, the greater wax moth *Galleria melonella* was used as an infection model for a better discrimination of possible attenuation effects in the three mutant strains. Experimental infections of *Galleria* larvae confirmed that deletions of *yqjG* or the *tatAC* genes do not affect virulence of *L. monocytogenes*. Killing curves of both mutant strains were similar to the wild type control experiment during which ~40% of the infected larvae survived (Fig. 2D). However, approximately 80% of the larvae infected with the  $\Delta$ *spoIIIJ* mutant were still viable seven days post infection (Fig. 2D). This demonstrates that SpoIIIJ but not YqjG has an impact on pathogenicity of *L. monocytogenes*.

### **Strains with defective bulk protein secretion**

For the identification of possible secretion defects caused by the absence of the *spoIIIJ*, *yqjG*, *tatAC*, *yukA*, *fliI* or *spoIIIAH* genes, all single mutant strains were grown in BHI broth at 37°C and culture supernatants were harvested at an optical density of  $OD_{600} = 1.5$ , corresponding to logarithmic growth phase (Fig. 1A-D). Extracellular proteins were isolated by TCA precipitation and exo-proteome samples were subjected to conventional one-dimensional SDS-PAGE. This revealed that the extracellular protein patterns of the  $\Delta spoIIIJ$ ,  $\Delta yqjG$ ,  $\Delta tatAC$ ,  $\Delta yukA$ ,  $\Delta fliI$  and  $\Delta spoIIIAH$  mutants were similar if not identical as compared to the wild type proteome sample (Fig. 3A and data not shown). The absence of secretion defects in these strains was later confirmed by two-dimensional SDS-PAGE (data not shown), which indicates that these genes individually do not impact on bulk protein secretion, at least under the conditions tested.

In contrast to this, depletion of YqjG from cells of the conditional  $\Delta spoIIIJ \Delta yqjG$  double mutant LMS101 visibly affected exo-proteome composition (Fig. 3B). Among the affected proteins was p60 (CwhA), well-known to be the most abundant protein in culture supernatants of *L. monocytogenes*. Reduction of p60 levels upon YidC depletion was confirmed by Western blotting using a p60 specific antiserum (data not shown). The most prominent effect on exo-proteome composition, however, was obtained upon SecA depletion from cells of the conditional *secA* mutant strain LMS104, which also affected secretion of p60 (Fig. 3C). This was not unexpected since SecA is the secretion ATPase of the Sec translocon responsible for bulk protein secretion (11, 17).

### **Identification of SecA-dependent extracellular proteins by proteomics**

Extracellular proteins isolated from SecA-expressing and SecA-depleted LMS104 cells (see experimental procedures section for a precise description of depletion conditions) were separated on two-dimensional PAGE gels using linear IEF strips (pH3-10, Fig. 4). 562 spots

were detected in total on these gels. Proteins spots were considered to be significantly accumulated or reduced upon SecA depletion when their spot volume was at least two times greater or lower in SecA-depleted LMS104 samples ( $n=3$ ,  $P<0.01$ ). These criteria led to the identification of 73 spots with a significant reduction after depletion of SecA. 166 spots with a significantly increased level were found, leaving 323 spots the amount of which was not or only insignificantly affected by SecA depletion. All 562 protein spots were excised from the gel and subjected to mass spectrometry. Identifications were considered as verified when the identification result was confirmed by a second identification obtained at the same gel position from an independent *L. monocytogenes* proteome sample in the course of this study. This yielded the identification of 107 verified protein spots (Fig. S1.1- 106, Fig. S2, Table S1) corresponding to 36 different listerial proteins. As shown in Table 4, ten proteins could be identified the amount of which is significantly reduced upon depletion of SecA. This included the listerial hemolysin listeriolysin O, the penicillin binding protein Lmo2039, several enzymes involved in degradation or modification of peptidoglycan (p45/Lmo2505, Lmo1521 and Lmo2591), as well as the chitin binding protein Lmo2467. Furthermore, the secretion of the quinol oxidase subunit QoxA, the lipoteichoic acid synthase LtaS, a protein with similarity to L-cystine binding proteins (TcyK) and the hypothetical protein Lmo0130 were dependent on SecA. All these proteins represent classical secretion substrates as illustrated by the presence of signal peptides and – in some cases – trans-membrane domains (TMD, Tab. 4). Eleven extracellular proteins were found the amount of which was not or only insignificantly affected by depletion of SecA: Among them are the actin assembly inducing ActA protein, the lipoteichoic acid permease LtaP, the penicillin binding protein B1 (Lmo1438), internalin C (InlC), the heme binding protein Hbp2, the chitinase ChiA, two hypothetical proteins (Lmo1752, Lmo2504), enolase (Eno), as well as p60 and OppA. With the exception of enolase, all these proteins contain signal sequences and thus represent classical secretion substrates. However, either their secretion does not require SecA at all, or the depletion

conditions used here were not stringent enough to reveal an effect on their translocation. The latter alternative most likely explains the absence of a statistically significant effect of SecA depletion in this experimental setup on secretion of p60, which is clearly affected under more stringent depletion conditions (Fig. 3C). Finally, 15 proteins were identified with an increased abundance after SecA depletion. With the exception of the newly identified listerial resuscitation promoting factor Lmo2522 (58, 59), all of them are typical cytosolic proteins and do not contain a signal peptide. The presence of cytosolic proteins in exoproteome samples could indicate an increased lysis of SecA-depleted cells as suggested by microscopical observation of SecA-depleted cells (data not shown). However, we cannot rule out the possibility that this is an artificial result caused by overrepresentation of background cytosolic proteins in proteome samples from secretion-deficient strains when same amounts of total proteins were loaded.

### **Identification of additional SecA substrates by azide inhibition experiments**

Since many of the known virulence factors escaped detection by mass spectroscopy, a set of complementary experiments was performed using protein-specific detection techniques. For inhibition of SecA, we used addition of sodium azide, which effectively inhibits SecA-dependent protein secretion (60). The main target of sodium azide is the SecA protein, since *secA* point mutations have been isolated which render *Escherichia coli* and *B. subtilis* azide-resistant (61-63). Likewise to *B. subtilis*, the *azi-1* mutation (SecAT128A) confers azide-resistant growth to *L. monocytogenes*, when the mutated *secAT128A* gene was present as a second ectopic copy (Fig. 5A). In order to test the impact of sodium azide (1 mM) on protein secretion, strains EGD-e (wt), LMS102 (*attB::P<sub>help</sub>-lacO-secA*) and LMS129 (*attB::P<sub>help</sub>-lacO-secAT128A*) were grown in BHI broth (supplemented with IPTG where required) at 37°C. When an OD<sub>600</sub> of 0.2 was reached, the culture was split. The cultivation of one half of the culture was continued in BHI broth, whereas 1 mM sodium azide (final concentration)

was added to the other half. When the cultures reached an OD<sub>600</sub> of 1.0, culture supernatants were harvested and extracellular proteins were precipitated using TCA. An obvious reduction of the levels of several extracellular proteins was visible in the supernatants of EGD-e and LMS102 cells on one-dimensional SDS-PAGE gels, indicating azide-dependent blockage of protein secretion. In contrast, no such azide effect was observed with the *azi-1* strain LMS129 (Fig. 5B). We have shown earlier that the most prominent supernatant protein (at ~60 kDa) under this condition corresponds to the major autolysin p60 (CwhA) (37) the secretion of which depends on SecA2 (20). Azide treatment led to a significant reduction of p60 levels in EGD-e (wt) and LMS102 (+*secA*) supernatants but not in LMS129 (+*secAT128A*, Fig. 5B), which was confirmed by immune-staining using a p60-specific antiserum (Fig. 5C). Likewise, secretion of the second SecA2-dependent autolysin MurA (NamA) was blocked by azide in EGD-e and LMS102 but not in the azide resistant *secAT128A* mutant strain (LMS129, Fig. 5C). The fact that a single mutation in the *secA* gene is sufficient to restore p60 and MurA secretion in the presence of azide implies that SecA contributes to secretion of SecA2 substrates and suggests that sodium azide does not block SecA2. To our knowledge, this is the first indication that SecA contributes to SecA2-dependent protein secretion in *L. monocytogenes*. Besides this, azide treatment blocked secretion of ActA, PlcA and PlcB – which were not identified as SecA-dependent proteins in the course of the SecA depletion experiments – and this effect was not seen in the *azi-1* strain (Fig. 5D). A surprising exception was InlA, the immunogenic detection of which in culture supernatants was facilitated by a replacement of the LPXTG motif by a C-terminal FLAG-tag. The presence of the additional, ectopically expressed *secA* genes in strains LMS134 (+*secA inlA'-flag*) and LMS135 (+*secAT128A, inlA'-flag*) enhanced secretion of InlA-FLAG as compared to the wild type control strain LMS132 (wt *inlA'-flag*), suggesting SecA-dependence. However, addition of azide did not block internalin A secretion in any of the three tested strains (Fig. 5D).

### **Identification of secretion defects caused by depletion of YidC activity**

Individual single knockouts of the YidC membrane insertase genes *spoIIIJ* and *yqjG* did not reveal any effects on the extracellular protein pattern in one- or two-dimensional PAGE experiments (Fig. 3A and data not shown). Therefore, YqjG was depleted from the conditional double mutant strain LMS101 in order to obtain cells lacking any activity of YidC-like proteins. Spots the amount of which was at least two-fold reduced upon YqjG-depletion in strain LMS101 were selected based on quantification of spot volumes and statistical evaluation (see above and experimental procedures). This revealed 14 protein spots with significantly reduced intensity after YqjG depletion in strain LMS101. These corresponded to six proteins: the quinol oxidase subunit QoxA, the major autolysin p60, an N-acetylmuramoyl-L-alanine amidase (Lmo1521), the chitinase ChiA (Lmo1883), the OppA protein (Lmo2196), as well as the binding protein of the cystine ABC transporter Lmo2349 (Fig. 6, Table 5). With the exception ChiA and OppA, all these proteins also require SecA for secretion according to our SecA depletion or inhibition experiments (Tab. 3, Fig. 5B-C). p60 and OppA are reported substrates of the SecA2 secretion pathway (20), even though conflicting results exist in the literature (22, 37). Thus, the YidC homologs of *L. monocytogenes* contribute to secretion of proteins that are routed via SecA or the accessory secretion ATPase SecA2. Lmo2349 shares high homology with TcyK (formely YtmK) from *B. subtilis* (77% identity) which was shown to contribute to L-cystin uptake (64, 65). Besides this, we identified eight extracellular proteins which were not affected by depletion of YqjG in strain LMS101 (Table 5, Table S1). This included proteins that depend on SecA, but also such ones which were SecA-independent. Possibly, their secretion depends on other pathways or they require stronger depletion conditions to be affected. Among the four proteins which accumulated in the culture supernatant upon YqjG depletion were cytosolic proteins such as the chaperones DnaK, GroEL and the glycolytic enzyme Gap, but also the penicillin binding protein Lmo2039.

### Secretion of putative Tat-substrates

A previous bioinformatic analysis had suggested that two listerial proteins possess a twin-arginine signal peptide: Lmo0367, encoding an iron depending peroxidase, and the  $\beta$ -ketoacyl-acyl carrier protein synthase II Lmo2201 (8). When the chromosomal *lmo0367* gene was C-terminally fused to a Strep-tag (strain LMS107), no expression signals could be obtained by Western blotting (data not shown). Hence, ectopically expressed Strep-tagged variants of Lmo0367 and Lmo2201 were constructed to ensure their immunogenic detection. The *lmo0367-strep* and *lmo2201-strep* alleles were expressed in wild type and  $\Delta$ *tatAC* mutant strains and their expression and their sorting into different cellular sub-compartments was analysed by Western blotting. Lmo0367-Strep was detected in the soluble cytosolic fraction of a cellular extract generated from strain LMS117 (*lmo0367-strep*), and also in the insoluble fraction containing membranes as well as cell wall material. However, it was not detected in the culture supernatant (Fig. 7A). This suggests that Lmo0367 is not released into the extracellular medium but is associated with the cell envelope. A similar result was obtained when secretion of Lmo0367-Strep was analysed in strain LMS126 ( $\Delta$ *tatAC lmo0367-strep*, Fig. 7A). This shows that the transport of Lmo0367-Strep into the cell envelope fraction is independent of the Tat pathway. This result was later confirmed using an antiserum recognizing EfeB (66), which is the *B. subtilis* homologue of Lmo0367. This antiserum cross-reacts with Lmo0367 and detected the endogenous, untagged Lmo0367 only in the cytosolic and the membrane fractions of *L. monocytogenes* EGD-e but not in the culture supernatant fraction (data not shown). Moreover, an identical sorting pattern of Lmo0367 was observed in the  $\Delta$ *tatAC* mutant strain LMS103 (data not shown). Likewise to what was observed for Lmo0367, the transport of Lmo2201-Strep into the cell envelope fraction also turned out to be clearly independent of TatAC (Fig. 7B).

## DISCUSSION

The results presented in this manuscript demonstrate that the house-keeping protein secretion routes via SecA and the two YidC homologs SpoIIIJ and YqjG are essential in *L. monocytogenes* and required for secretion of many extracellular proteins. The Tat pathway, in contrast, is not required for viability, virulence or protein secretion under the conditions tested here. This was somewhat unexpected, since one of its putative substrates, Lmo0367, is supposed to contribute to iron uptake (see below), and thus might be critical for growth and survival inside eukaryotic hosts.

Out of the 36 proteins, which we have identified in exoproteome samples of *L. monocytogenes* (Tab. 4, Tab. S1), 21 have a signal peptide (17). This indicates that these proteins were real secretory proteins. Secretion of ten of them in fact depends on SecA (Fig. 4 and Tab. 4). This list contains the essential penicillin binding protein Lmo2039 (PBP B2) (67) and the lipoteichoic acid synthase LtaS, the knock-out of which causes severe growth and division defects (68). This finding on its own illustrates the reason for the essentiality of SecA, even though our experiments only detect proteins released into the extracellular space. Proteins that are either covalently linked to the cell wall or the cell membrane or even inserted into it and also depend on SecA are not covered by our approach. Out of the major listerial virulence factors, we only found the pore-forming hemolysin listeriolysin O, which is absolutely essential for virulence (69), as a SecA-dependent protein in the 2D PAGE approach. The chitin-binding protein Lmo2467 was also among the clearly SecA-dependent proteins (Fig. 4 and Tab. 4). Even though mammalian cells do not synthesize chitin, mutants in *lmo2467* and the chitinase *chiA* are attenuated in mice (70). This apparent contradiction was solved by the finding that ChiA contributes to bacterial survival in eukaryotes by repression of host innate immunity (71). Using azide as SecA inhibitor, we could furthermore demonstrate that secretion of the virulence factors ActA and the phospholipases PlcA and

PlcB requires SecA as well. This shows that protein secretion via the SecA route directly contributes to vacuolar escape, intracellular motility and cell-to-cell spread of *L. monocytogenes* and verifies the concept of SecA inhibitors as anti-infective drug candidates. Why secretion of InlA appears SecA-independent in this assay is not clear since it possesses a classical Sec-type signal sequence.

15 proteins were found in the secretome samples which do not possess a signal peptide. However, the amounts all of them (with the exception of the glycolytic enzyme enolase) were clearly increased upon SecA depletion. When SecA is depleted, the bacteria tend to lyse (data not shown), so that cytoplasmic proteins possibly could contaminate the secretome samples. The amount of enolase, however, did not increase upon SecA depletion, indicating that the presence of enolase in the culture supernatant is not caused by cell lysis. This would be in good agreement with a similar phenomenon in *B. subtilis*, where enolase is found in culture supernatants independently from lysis (72). The underlying mechanism is not understood, but depends on a central helix in the enolase molecule and is termed non-classical protein secretion (72).

Mutants lacking *secA2* still secrete background amounts of p60 (24, 37), suggesting that other pathways contribute to p60 translocation. We show that secretion of two SecA2 substrates also depends on SecA, which can have two possible explanations: (i) SecA inactivation might affect membrane insertion of components of the Sec translocon, or (ii) SecA acts in concert with SecA2 to translocate SecA2 substrates. Presently it is not known, whether SecA2 alone is sufficient to energize secretion of its genuine substrates or whether it requires assistance of SecA (26, 73). *In vitro* experiments with purified components are needed to answer this question. The substrate spectrum of the *L. monocytogenes* SecA route also overlaps partially with the YidC pathway: We identified three proteins (p60, Lmo1521, and TcyK) affected by depletion (or inhibition) of SecA and YidC. With the exception of QoxA, none of the YidC-dependent proteins contain transmembrane segments that could

explain their dependency on SpoIIIJ and YqjG. Thus, it seems rather unlikely that these are direct effects. Possibly, mis-incorporation of other secretion-relevant proteins such as the secretion pore components into the membrane upon YidC depletion interferes with secretion of SecA substrates. That this effect is only observed with a subset of the SecA-dependent secreted exo-proteins may indicate a gradual sensitivity of the different substrate proteins to different activity levels of the Sec translocon. Remarkably, deletion of *spoIIIJ* but not of *yqjG* affected survival of *L. monocytogenes* in the insect model. This suggests that the substrate specificity of SpoIIIJ must be somewhat extended and that SpoIIIJ-dependent proteins are of special importance for general fitness or virulence in the insect host.

None of the two putative listerial Tat substrates Lmo0367 and Lmo2201 (8) was Tat-dependent in our experiments. Lmo0367 is homologous to the well-known *B. subtilis* Tat-substrate EfeB (formerly YwbN, 49.8% identity), which is a hemoprotein required for the oxidation of ferrous to ferric iron for uptake by the EfeUO transporter (66). Lmo2201 is equivalent to *B. subtilis* FabF of (70.5% identity), a component of the cytoplasmic  $\beta$ -ketoacyl- (acyl carrier protein) synthase complex and has no transmembrane domain (17). Thus, its classification as a putative Tat substrate has to be considered a misannotation. That Lmo2201 nevertheless was found in the membrane fraction (Fig. 7B) suggests that it might be tightly associated with the cytoplasmic face of the phospholipid bilayer. EfeB of *B. subtilis* is attached to the membrane via the EfeU permease component (66), but also released into the extracellular milieu (74). Lmo0367 has never been detected in exo-proteome samples of *L. monocytogenes*, but is present in membrane fractions, as in *B. subtilis*. However, even the membrane targeting of Lmo0367 was Tat-independent. Transcription of the *L. monocytogenes* *tatAC* operon is induced during iron starvation (75), but also clearly transcribed during logarithmic growth phase in plain BHI broth (39), the condition that was chosen here. We have added 2,2'-dipyridyl to the growth medium to deplete the iron pool, but have not seen any effects of TatAC on secretion or membrane targeting of Lmo0367 (data not shown).

Possibly, the Tat-pathway is not functional in *L. monocytogenes* or more specific conditions are required for its activity. This would be in good agreement with the repeated observation that deletion of *tatAC* does not cause attenuation of virulence in different infection models (this work) (39).

Taken together, the results shown here represent the first study which experimentally analyses genome sequence-based bioinformatic predictions on protein secretion via the three house-keeping translocation routes in *L. monocytogenes*. More detailed experiments are required to study their impact on composition of the membrane proteome or on protein secretion during infection.

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## FIGURE LEGENDS

**Figure 1:** Growth of *L. monocytogenes* secretion mutants.

(A) Growth of *L. monocytogenes* single mutants lacking either *yqjG* (LMS6) or *spoIIIJ* (LMS100) in BHI broth at 37°C. (B) Growth of the *L. monocytogenes*  $\Delta$ *tatAC* mutant strain LMS103. (C) Growth of the conditional *spoIIIJ yqjG* double mutant LMS101 in BHI broth containing or not containing 1 mM IPTG. LMS101 cells were pre-grown in BHI containing 1 mM IPTG, transferred to BHI broth without IPTG, and cultivated to stationary phase to efficiently deplete the cells for YqjG. YqjG-depleted LMS101 cells were then used to inoculate cultures containing 1 mM IPTG or lacking the inducer and growth was monitored in hourly intervals. The wild-type strain EGD-e was included for comparison. (D) Growth of the conditional *secA* mutant strain LMS104 in BHI broth at 37°C either containing 1 mM IPTG or lacking IPTG. Depletion was done as described for LMS101, strain EGD-e was included as a control. (E) Western-blot with cellular extracts of the conditional *secA* mutant strain LMS104 grown in the presence of different IPTG concentrations. SecA was visualized using an anti-SecA antiserum generated against SecA of *Bacillus subtilis* (45). *B. subtilis* SecA was included as a positive control.

**Figure 2:** Virulence assays of *L. monocytogenes* secretion mutants.

(A) Plaque formation assay with *L. monocytogenes* mutants lacking *spoIIIJ* (LMS100), *yqjG* (LMS6), or the *tatAC* genes (LMS103) using the mouse embryo fibroblast cell line 3T3 L1. Infection was performed as described in the Materials and Methods section. Plaques became visible at day three post infection after staining of live 3T3 cells using an agarose overlay containing 0.01% neutral red. Uninfected 3T3 cells were included for control (-). (B) Intracellular growth kinetics of the same set of *L. monocytogenes* mutant strains in HeLa cells. The HeLa cells were infected with a multiplicity of infection of 25 at time point -2 h,

whereupon the infection was carried out for one hour. Extracellular bacteria were killed by gentamicin treatment during another one hour incubation step. At the given time points the infected HeLa cells were lysed and serial dilutions were plated on BHI agar plates to count intracellular bacteria. Standard deviations were calculated from an experiment performed in triplicate. (C) Intracellular growth of *L. monocytogenes* secretion mutant strains in J774 mouse macrophages. Bacterial strains were used to infect mouse macrophages at a MOI of 0.1667 and intracellular growth was recorded based on CFU determination. Average values and standard deviations were calculated from an experiment performed in triplicate. (D) Survival curves of *Galleria melonella* larvae infected with *L. monocytogenes* secretion mutants. Mock infections using NaCl solution were included as negative control. Results represent mean values of at least three independent experiments with a total of 60 larvae per treatment.

**Figure 3:** Secretome profiles of *L. monocytogenes* secretion mutants

(A-C) SDS-PAGE showing separated secretome samples of *L. monocytogenes* secretion mutants. Strains lacking *spoIIIJ* (LMS100), *yjgG* (LMS6) and *tatAC* (LMS103) were grown in BHI broth at 37°C and culture supernatants were harvested at an optical density of 1.5 (A). Depletion of YidC (B) and SecA proteins (C) in strains LMS101 and LMS104, respectively, were performed as described in the legend to figure 1 and depleted cells of LMS101 and LMS104 were used to inoculate cultures containing 5 or 100 µM IPTG.

**Figure 4:** Effect of SecA depletion on the exoproteome of *L. monocytogenes*.

The SecA depletion strain LMS104 was cultivated in BHI broth at 37°C either in the absence (red) or the presence of 100 µM IPTG (green) and extracellular proteins were isolated from the culture supernatants (see experimental procedures for depletion details). Proteome samples were separated by two-dimensional SDS-PAGE using IPG strips with a linear pH

range of pH3-10 and gel images were warped using the Decodon Delta2D software package. Proteins were excised from the gels and analysed by mass spectrometry. Spot volumes were quantified and proteins with a statistically significant reduction or induction ratio of more than two-fold are labelled in green or red, respectively (n=3,  $P < 0.01$ ). Identified proteins that do not show statistically significant induction/reduction ratios of more than two-fold are labelled in white. Numbers used for designation of protein spots correspond to the unique gene identifiers of *L. monocytogenes* EGD-e (e.g. 0644 = Lmo0644). Detailed protein identification results and fold changes for all identified protein spots can be found in Tab. S1.

**Figure 5:** Effect of sodium azide on growth and protein secretion of *L. monocytogenes*.

(A) Growth of *L. monocytogenes* strains on BHI agar containing 1 mM sodium azide. Strains EGD-e (wt), LMS102 (+*secA*, *attB*::*P<sub>help</sub>*-*lacO-secA*) as well as LMS129 (+*azi-1*, *attB*::*P<sub>help</sub>*-*lacO-secA**T128A*) were streaked on BHI plates containing 1 mM IPTG as well as 1 mM sodium azide. The plate was incubated at 37°C for 40 h and photographed. (B) SDS-PAGE gel of secretome samples of the same set of strains grown in BHI broth containing or not containing 1 mM sodium azide at 37°C (for details see text). 1 mM IPTG was added to the LMS102 and LMS129 cultures to allow expression of SecA proteins. (C) Effect of azide treatment on secretion of p60 (upper panel, Western blot) and MurA (lower panel, zymography) in the same set of strains under identical conditions. (D) Effect of azide treatment on secretion of ActA, PlcA, PlcB and InlA. ActA, PlcA and PlcB in extracellular extracts of the same set of strains as in panel A were immune-stained using their respective antisera. InlA was detected as a truncated version lacking the C-terminal LPXTG motif and bearing a C-terminal FLAG-tag. The strains used in this experiment were LMS132 (wt *inlA'*-*flag*), LMS134 (+*secA inlA'*-*flag*) and LMS135 (+*azi-1, inlA'*-*flag*).

**Figure 6:** Contribution of YidC proteins SpoIIIJ and YqjG to exoproteome composition of *L. monocytogenes*.

Extracellular proteins of strain LMS101 ( $\Delta spoIIIJ \Delta yqjG attB::P_{help-lacO-yqjG}$ ) were collected from the culture supernatants after growth in the presence of 100  $\mu$ M IPTG (induction of YqjG, green) and 5  $\mu$ M IPTG (depletion of YqjG, red) and separated by two-dimensional SDS PAGE using IPG strips with a linear pH range of pH3-10. Gel images were false-colored and brought to congruency by warping using the Delta2D software package (Decodon). Protein spots with a statistically significant induction/reduction ratio of more than two-fold were labelled in green or red, respectively ( $n=3$ ,  $P<0.01$ ). Identified proteins with unchanged abundances are labelled in white. Numbers used for designation of protein spots correspond to the unique gene identifiers of *L. monocytogenes* EGD-e (e.g. 1438 = Lmo1438). Detailed protein identification results and fold changes for all identified protein spots can be found in Tab. S1.

**Figure 7:** Subcellular sorting of Lmo0367 and Lmo2201

(A) Western blot showing expression and secretion of Lmo0367-Strep in the wild type (strain LMS117) and the  $\Delta tatAC$  background of *L. monocytogenes* (strain LMS126). Cells were grown in BHI broth containing 1 mM IPTG up to an optical density of 1.0, harvested and cellular fractions were isolated as described in Materials and Methods. (B) Western blot showing expression of Lmo2201-Strep in the wild type (LMS113) and the  $\Delta tatAC$  mutant strain LMS115. The experiment was performed as described for panel A. The wild-type strain EGD-e served as a negative control (co). Strep-tagged proteins were immune-stained using an antiserum recognizing the Strep-Tag.

**Table 1:** Protein secretion systems encoded by the *L. monocytogenes* EGD-e genome<sup>1</sup>.

transport system	components	genes	substrates	references			
<i>Sec system</i>							
• pore components	SecY	<i>lmo2612</i>	Sec substrates				
	SecE	<i>lmo0245</i>					
	SecG	<i>lmo2451</i>					
	• associated proteins	SecDF			<i>lmo1527</i>	(40)	
		YajC			<i>lmo1529</i>		
		SecA			<i>lmo2510</i>		
	• general ATPase	SecA2			<i>lmo0583</i>	specific Sec substrates	(43)
	• accessory ATPase	FtsY			<i>lmo1803</i>		
	• signal recognition particle	YlxM			<i>lmo1802</i>	cotranslational Sec transport	
		Ffh			<i>lmo1801</i>		
SRP 4.5S RNA <sup>2</sup>							
• membrane integrases		SpoIIJ	<i>lmo2854</i>	membrane proteins			
YqjG	<i>lmo1379</i>						
<i>twin-arginine transport</i>							
• pore components	TatA	<i>lmo0362</i>	folded substrates	(39)			
	TatC	<i>lmo0361</i>					
<i>flagellum exporter</i>							
• pore components	FlhBA	<i>lmo0679-0680</i>	flagellar proteins	(76)			
	FliO	<i>lmo0682</i>					
	FliPQR	<i>lmo0676-0678</i>					
• ATPase complex	FliH	<i>lmo0715</i>		(77)			
	FliI	<i>lmo0716</i>					
<i>fimbrial exporter</i>							
• ATPase	ComGA	<i>lmo1347</i>	prepilins ComGC-GG	(36)			
• pore component	ComGB	<i>lmo1346</i>					
• prepilin peptidase	ComC	<i>lmo1550</i>					
<i>holins</i>							
• TcdE type	Lmo0128	<i>lmo0128</i>	phage endolysins	(78)			
• φA118 type	Lmo2279	<i>lmo2279</i>					
<i>WXG100 system</i>							
• pore components	Lmo0057-60	<i>lmo0057-60</i>	WXG100 proteins	(38)			
• ATPase	YukAB	<i>lmo0061</i>					

<sup>1</sup> Classification of secretion systems is according to a genome survey published by Desvaux and Hébraud (8). <sup>2</sup> There is no *lmo* number available for the 4.5S RNA gene, which is located between the *lmo2710* and *lmo2711* open reading frames.

**Table 2:** Plasmids and strains used in this study

name	relevant characteristics	source*/reference
<b>plasmids</b>		
pAUL-A	<i>erm lacZα</i>	(79)
pET19b- <i>secA</i>	<i>bla lacI his<sub>6</sub>-secA</i>	(80)
pIMK2	P <sub>help</sub> <i>neo</i>	(43)
pIMK3	P <sub>help</sub> - <i>lacO lacI neo</i>	(43)
pMAD	<i>bla erm bgaB</i>	(44)
pUC19	<i>bla lacZα</i>	Invitrogen
pSH183	<i>bla erm bgaB ΔspoIIIAH (lmo0791)</i>	this work
pSH184	<i>bla erm bgaB ΔyukA (lmo0061)</i>	this work
pSH188	<i>bla erm bgaB yqjG region</i>	this work
pSH189	<i>bla erm bgaB ΔyqjG (lmo1379)</i>	this work
pSH199	P <sub>help</sub> - <i>yqjG neo</i>	this work
pSH208	P <sub>help</sub> - <i>lacO-yqjG lacI neo</i>	this work
pSH341	<i>bla erm bgaB ΔspoIIIJ (lmo2854)</i>	this work
pSH351	<i>bla tatAC genomic region</i>	this work
pSH352	P <sub>help</sub> - <i>lacO-secA lacI neo</i>	this work
pSH353	<i>bla ΔtatAC</i>	this work
pSH356	<i>bla secA genomic region</i>	this work
pSH357	<i>bla ΔsecA</i>	this work
pSH360	<i>bla erm bgaB ΔtatAC (lmo0362 lmo0361)</i>	this work
pSH361	<i>bla erm bgaB ΔsecA (lmo2510)</i>	this work
pSH366	<i>lmo2201-strep erm</i>	this work
pSH367	<i>lmo0367-strep erm</i>	this work
pSH376	P <sub>help</sub> - <i>lacO-lmo2201-strep lacI neo</i>	this work
pSH377	P <sub>help</sub> - <i>lacO-lmo0367-strep lacI neo</i>	this work
pSH380	P <sub>help</sub> - <i>lacO-lmo0367-strep<sup>2</sup> lacI neo</i>	this work
pSH383	P <sub>help</sub> - <i>lacO-secAT128A lacI neo</i>	this work
pSH386	<i>inlA`-flag erm</i>	this work
<b><i>L. monocytogenes</i> strains</b>		
EGD-e	wild type, serovar 1/2a strain	(41)
LMS3	Δ <i>fliI</i>	(37)
LMS4	Δ <i>yukA</i>	pSH184 ↔ EGD-e
LMS5	Δ <i>spoIIIAH</i>	pSH183 ↔ EGD-e
LMS6	Δ <i>yqjG</i>	pSH189 ↔ EGD-e
LMS15	Δ <i>yqjG attB::P<sub>help</sub>-lacO-yqjG lacI neo</i>	pSH208 → LMS6
LMS100	Δ <i>spoIIIJ</i>	pSH341 ↔ EGD-e
LMS101	Δ <i>spoIIIJ ΔyqjG attB::P<sub>help</sub>-lacO-yqjG lacI neo</i>	pSH341 → LMS15
LMS102	<i>attB::P<sub>help</sub>-lacO-secA lacI neo</i>	pSH352 → EGD-e
LMS103	Δ <i>tatAC</i>	pSH360 ↔ EGD-e
LMS104	Δ <i>secA attB::P<sub>help</sub>-lacO-secA lacI neo</i>	pSH361 ↔ LMS102
LMS105	<i>lmo2201-strep erm</i>	pSH366 → EGD-e
LMS107	<i>lmo0367-strep erm</i>	pSH367 → EGD-e
LMS113	<i>attB::P<sub>help</sub>-lacO-lmo2201-strep lacI neo</i>	pSH376 → EGD-e
LMS115	Δ <i>tatAC attB::P<sub>help</sub>-lacO-lmo2201-strep lacI neo</i>	pSH376 → LMS103
LMS117	<i>attB::P<sub>help</sub>-lacO-lmo0367-strep<sup>2</sup> lacI neo</i>	pSH380 → EGD-e
LMS126	Δ <i>tatAC attB::P<sub>help</sub>-lacO-lmo0367-strep<sup>2</sup> lacI neo</i>	pSH360 ↔ LMS117
LMS129	<i>attB::P<sub>help</sub>-lacO-secAT128A lacI neo</i>	pSH383 → EGD-e
LMS132	<i>inlA`-flag erm</i>	pSH386 → EGD-e
LMS134	<i>inlA`-flag erm attB::P<sub>help</sub>-lacO-secA lacI neo</i>	pSH386 → LMS102
LMS135	<i>inlA`-flag erm attB::P<sub>help</sub>-lacO-secAT128A lacI neo</i>	pSH386 → LMS129

\* The arrow (→) stands for a transformation event and the double arrow (↔) indicates gene deletions obtained by chromosomal insertion and subsequent excision of pMAD plasmid derivatives (see experimental procedures for details).

**Table 3:** Oligonucleotides used in this study.

<b>name</b>	<b>sequence (5'→3')</b>
SHW1	CGGGATCCAGCACAGTTAGATTCAGTTAGG
SHW2	ATATGTCGACTTGAATTCCAATTCATTGTACAG
SHW3	ATATGTCGACGGGAATACACCAATAACTTTCAG
SHW4	GCATGCCATGGGCGGTAATCCCCATTTTCATC
SHW7	ATATGTCGACAAGTGAGAGATATAACTGCGC
SHW8	GCATGCCATGGCACCAAGCTCATTTGCCATCC
SHW21	TCTCGAATTCTTTTCGATATTAACCTAGTGCC
SHW22	ATAAGAATGCGGCCGCCTTATTTTGCTTCTGTATTTGTTG
SHW23	ATAAGAATGCGGCCGCTTGAAAGGAGCTGTTAATGATGG
SHW24	ATATGTCGACAGCCGGTCAAATCCTTCAGC
SHW45	CGGGATCCTCCTCTTGAATGGATGTTTCC
SHW46	ATATGTCGACCAATGTTTCATTTCCCTCCTAAATAG
SHW47	ATATGTCGACTAAAAGAAACCGCAACCTCG
SHW48	GCATGCCATGGCCCACTGACTCGTCTAGCTCG
SHW99	CATGGAAAAGGATCCAAAGAAAAATATTATTTAATTAGCG
SHW100	AATGGGATCGTCGACTTATTTTCTTTCTTTTGTCTTC
SHW116	CAGCTATGACCATGATTACGC
SHW388	TATCGGATCCGTCAATTTGTTGTTTATACTTTAAAG
SHW390	ATATGTCGACTTATGTTTCATTATCTTTAGACGAG
SHW411	ATATGTCGACAACTAAACCAAAGGAGCTACG
SHW412	GCATGCCATGGTTAAGTAAATTTGATGGCCTGC
SHW413	ATTTTAGGATCCATTTTCGATTTCTCCATTCTT
SHW414	AAATGGGATCCTAAAATGAATTATTTATACTAAAAGCTC
SHW415	ATATGTCGACGAAGTAACAGAACCGATTTCGAG
SHW416	GCATGCCATGGCGTACTTCAATTTCAATATCGCC
SHW417	AATGGGATCCTAGATTGAAACAATTTAAAAAGCGC
SHW418	TCTAGGATCCCATTTAAATTCTCCTCTACTGTT
SHW419	GCATGCCATGGCTGGACTATTGAAAAAAATTTTG
SHW420	ATATGTCGACCTATGCTTCTTTACCATGACAATTT
SHW439	ATGAGGCTTATTCTGACGGCG
SHW440	TCATTTTTCGAACTGCGGGTGGCTCCAAAGTAGTTTTCTCCAAAATTTCTC
SHW441	CAAACCTCAATCGGCGACGCG
SHW442	TCATTTTTCGAACTGCGGGTGGCTCCAGTCTTCTATTCTTTTAAATACTAAAG
SHW463	CCGGGCTGCAGGAATTCGAGCTCGGTACCC
SHW464	GAAACCCATGGATAAGAGAAGAGTAGTTG
SHW465	GAAACCCATGGCAGATAAGAAGAGCGAAAATC
SHW474	CCACCCGCAGTTCGAAAAAACGACACTGGAGAAGCGGATG
SHW475	GAACTGCGGGTGGCTCCAATCACGCAAAACCCCACTC
SHW490	CATGTGGTTGCTGTCAATGAATACTTAGCTC
SHW491	TCATTGACAGCAACCACATGCACCCCTTC
SHW497	GTGACGCAGCCACTTAAGGC
SHW498	TTACTTGTTCATCGTCATCCTTGTAAATCAGGGTCATAAGCGTTCATTGTAC

**Table 4:** Abundance of proteins detected in in extracellular proteomes of strain LMS104 after depletion of SecA as shown in Fig. 4.

ORF ID	name	function	signal sequence <sup>1</sup>	TMD <sup>1</sup>
<i>reduced abundance</i> <sup>2</sup>				
Lmo0013	QoxA	AA3-600 quinol oxidase subunit II	Sec-SPII	3
Lmo0130		hypothetical protein	Sec-SPI	-
Lmo0202	Llo	listeriolysin O	Sec-SPI	-
Lmo0927	LtaS	lipoteichoic acid synthase	Unc-SP	5
Lmo1521		N-acetylmuramoyl-L-alanine amidase	Sec-SPI	-
Lmo2039	PBP B2	penicillin-binding protein B2	Sec-SPI	-
Lmo2349	TcyK	L-cystine transport system substrate-binding protein	Sec-SPII	-
Lmo2467		chitin binding protein	Sec-SPI	-
Lmo2505	p45	peptidoglycan lytic protein	Sec-SPI	-
Lmo2591		similar to N-acetylmuramoyl-L-alanine amidase	Sec-SPI	-
<i>abundance unaffected</i> <sup>2</sup>				
Lmo0204	ActA	actin-assembly inducing protein	Sec-SPI	2
Lmo0582	p60	major autolysin	Sec-SPI	-
Lmo0644	LtaP	lipoteichoic acid primase	Unc-SP	5
Lmo1438	PBP B1	penicillin binding protein B1	Sec-SPI	-
Lmo1752		hypothetical protein	Sec-SPI	-
Lmo1786	InlC	internalin C	Sec-SPI	-
Lmo1883	ChiA	chitinase	Sec-SPI	-
Lmo2185	Hbp2	heme binding protein	Sec-SPI	-
Lmo2196	OppA	similar to pheromone ABC transporter binding protein	Sec-SPII	-
Lmo2455	Eno	enolase	-	-
Lmo2504		hypothetical protein, similar to cell wall binding proteins	Sec-SPI	-
<i>increased abundance</i> <sup>2</sup>				
Lmo0539		tagatose 1,6-diphosphate aldolase	-	-
Lmo0811		carbonic anhydrase	-	-
Lmo1055	PdhD	dihydrolipoamide dehydrogenase	-	-
Lmo1439	Sod	superoxide dismutase	-	-
Lmo1473	DnaK	molecular chaperone DnaK	-	-
Lmo1570	PykA	pyruvate kinase	-	-
Lmo2068	GroEL	molecular chaperone GroEL	-	-
Lmo2206	ClpB	ATP-dependent Clp protease ATP-binding subunit ClpB	-	-
Lmo2367	Pgi	glucose-6-phosphate isomerase	-	2
Lmo2459	Gap	glyceraldehyde 3-phosphate dehydrogenase	-	-
Lmo2522		SpsA/YocH type resuscitation promoting factor	Sec-SPI	-
Lmo2620	RplE	ribosomal protein L5	-	-
Lmo2653	TufA	elongation factor Tu	-	-
Lmo2654	Fus	elongation factor G	-	-
Lmo2785	Kat	catalase	-	-

<sup>1</sup> according to (17), abbreviations: Sec-SPI – Type I Sec signal sequence, Sec-SPII – Type II Sec signal sequence, Unc-SP – uncleaved signal peptide. <sup>2</sup> reduced abundance: spot volumes at least two-fold reduced, increased abundance: spot volumes at least two-fold increased, abundance unaffected: all other protein spots. Fold changes of all individual protein spots can be found in Tab. S1.

**Table 5:** Abundance of proteins detected in in extracellular proteomes of strain LMS101 after depletion of YidC activity as shown in Fig. 6.

ORF ID	name	function	signal sequence <sup>1</sup>	TMD <sup>1</sup>
<i>reduced abundance</i> <sup>2</sup>				
Lmo0013	QoxA	AA3-600 quinol oxidase subunit II	Sec-SPII	3
Lmo0582	p60	major autolysin	Sec-SPI	-
Lmo1521		N-acetylmuramoyl-L-alanine amidase	Sec-SPI	-
Lmo1883	ChiA	chitinase	Sec-SPI	-
Lmo2196	OppA	similar to pheromone ABC transporter binding protein	Sec-SPII	-
Lmo2349	TcyK	L-cystine transport system substrate-binding protein	Sec-SPII	-
<i>abundance unaffected</i> <sup>2</sup>				
Lmo0202	Llo	listeriolysin O	Sec-SPI	-
Lmo0927	LtaS	lipoteichoic acid synthase	Unc-SP	5
Lmo1438	PBP B1	penicillin binding protein B1	Sec-SPI	-
Lmo2185	Hbp2	heme binding protein	Sec-SPI	-
Lmo2504		hypothetical protein, similar to cell wall binding proteins	Sec-SPI	-
Lmo2505	p45	peptidoglycan lytic protein	Sec-SPI	-
Lmo2522		SpsA/YocH type resuscitation promoting factor	Sec-SPI	-
Lmo2591		similar to N-acetylmuramoyl-L-alanine amidase	Sec-SPI	-
<i>increased abundance</i> <sup>2</sup>				
Lmo1473	DnaK	molecular chaperone DnaK	-	-
Lmo2039	PBP B2	penicillin-binding protein B2	Sec-SPI	-
Lmo2068	GroEL	molecular chaperone GroEL	-	-
Lmo2459	Gap	glyceraldehyde 3-phosphate dehydrogenase	-	-

<sup>1</sup> according to (17), abbreviations: Sec-SPI – Type I Sec signal sequence, Sec-SPII – Type II Sec signal sequence, Unc-SP – uncleaved signal peptide. <sup>2</sup> reduced abundance: spot volumes at least two-fold reduced, increased abundance: spot volumes at least two-fold increased, abundance unaffected: all other protein spots. Fold changes of all individual protein spots can be found in Tab. S1.