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Contribution of the multiple Type I signal peptidases to the secretome of *Listeria monocytogenes*: Deciphering their specificity for secreted exoproteins by exoproteomic analysis

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ABSTRACT

As commonly seen in monoderm bacteria, *Listeria monocytogenes* possesses multiple membrane-bound signal peptidases of Type I (SPases I) called SipX, SipY and SipZ. In order to decipher their respective contribution in an integrated and global view, the complement of the secretome corresponding to the exoproteome was resolved by two-dimensional gel electrophoresis (2-DE). This was performed for *L. monocytogenes sipX*$^-$, *sipY*$^-$, *sipZ*$^-$ single mutants, as well as for Δ*sipXY* and Δ*sipYZ* double mutants, and then compared to that of the wild type strain. Remarkably, the amounts of listeriolysin O (LLO), phosphatidylcholine phospholipase C (PlcB) and zinc metalloproteinase Mpl in the extracellular milieu were significantly decreased upon inactivation of SipZ. For the majority of the Sec-secreted exoproteins identified, protein secretion was not affected by the inactivation of one or two of the SPases I, supporting the concept that the three SPases I have overlapping specificities for the cleavage of the signal peptides. The current study reveals that the role of SipZ as the major SPase I of *L. monocytogenes* applies only to a small subset of the secreted exoproteins. Rather than absolute, the notion of major and minor SPases thus appears to be relative. In addition to new insight into bacterial physiology, this investigation of the contribution of the SPases I to the exoproteome of *L. monocytogenes* paves the way for further characterization of other complements of the secretome under various environmental conditions.

Biological significance
L. monocytogenes encodes three orthologous signal peptidases of Type I (SPases I). SipZ improves the secretion efficiency for a subset of extracellular virulence factors. Multiple

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SPases I are functionally redundant for the majority of the Sec-secreted exoproteins of *L. monocytogenes*. The concepts of major and minor SPases are not absolute but relative.

1. Introduction

Listeria monocytogenes is a monoderm bacterium and an opportunistic foodborne pathogen, which essentially infects immunocompromised individuals. Listeriosis is a rare but serious disease characterized by septicemia, encephalitis, meningitis, neonatal sepsis or abortion [1]. In recent years, the number of listeriosis cases is on the increase in several European countries [2,3]. This ubiquitous species can survive and multiply in adverse environments, which confer an ability to persist in the food industry [4–9]. Infectious cycle of *L. monocytogenes* is now well characterized and involves several key virulence factors [10–13]. In brief, internalins (InlA and InlB) mediate bacterial adhesion to the host cell and then internalization. The listeriolysin O (LLO) and the phosphatidylinositol phospholipase (PlcA) lyse the phagosome membrane to liberate the bacterium into the cytosol. Cell-to-cell spreading involves ActA, which promotes intracellular movement through polymerization of actin, and the two phospholipases C (PlcA and PlcB), which lyse the two-membrane vacuole. As a leitmotif in the field of bacterial pathogenesis, these virulence factors require the involvement of protein secretion systems to be translocated across the cytoplasmic membrane in monoderm bacteria [14,15].

From proteogenomic analysis of the secretome [16,17], seven protein secretion systems have been uncovered in *L. monocytogenes* and the Sec pathway appeared prevalently used by the vast majority of the predicted secreted proteins (96%), including all key virulence factors. While somehow misunderstood by part of the scientific literature [14,15], the secretome is a very useful and powerful concept that by definition considered the protein secretion routes at the cellular level in an integrated and global view [18–21]; consequently, the secretome includes both the secretion machineries (*e.g.* translocation mechanisms, post-translational, post-translocational modifications) and their cognate substrates (*i.e.* the secreted proteins). The exoproteome (*i.e.* the extracellular proteome) corresponds to the subset of proteins present in the extracellular milieu (*i.e.* the exoproteins) [14], it is just one of the different complements of the secretome [18,22] but also the most commonly investigated [23].

In the Sec system, the translocon is constituted of the protein-conducting channel SecYEG and SecDF-YajC, which modulates the efficiency of translocation [16,18,24]. The ATPase SecA provides the energy for translocation of secreted proteins and forms the translocase together with the SecYEG-DF-YajC translocon, while the accessory SecA2 allows the specific secretion of a protein subset in some monoderm bacteria (archetypal Gram-positive bacteria) such as *L. monocytogenes* [25–27]. Sec-secreted proteins can either be (i) anchored to the cytoplasmic membrane, (ii) associated with the cell wall, or (iii) released into the extracellular milieu and even beyond, *i.e.* into a host cell [15,28]. Once translocated, most secreted proteins are subjected to further post-translational modifications, one of the first being the cleavage of their N-terminal signal peptide (SP) by a specific signal peptidase of Type I (SPase I) [29,30]. While diderm-LPS bacteria (archetypal Gram-negative bacteria) have typically only one SPase I, the occurrence of multiple paralogues is commonly observed in monoderm bacteria [29]. However, few proteomic investigations have been dedicated to the contribution of these multiple SPases I to the secretome [19,31].

According to the latest and most updated proteogenomic analysis in *L. monocytogenes*, 69 proteins out of the 714 Sec-secreted proteins exhibiting an N-terminal SP were predicted as located in the extracellular milieu, *i.e.* GO:0005576 as defined by the gene ontology (GO) [17]. Following two-dimensional gel electrophoresis (2-DE) analysis of the exoproteome, a total of 16 Sec-secreted exoproteins could be experimentally identified [32,33]. In *L. monocytogenes*, three SPases I have been identified as involved in the maturation of the pre-proteins, namely SipX, SipY and SipZ [16,34]. The *sip* genes belong to a cluster where the promoter for *sipX* and *sipY* is constitutively activated, whereas the promoter for *sipZ* is temporally controlled [35]. Focusing on some key virulence factors of *L. monocytogenes*, the contribution of the three SPases I to protein secretion and at different stages of the infectious process was investigated [34] but only a subset of 5 virulence factors including only 2 exoproteins were considered, *i.e.* LLO and PlcB. SipZ appeared as the prevalent SPase I involved in the maturation of LLO, PlcB, InlA, InlB and ActA. The efficiency of SipX to mature LLO and PlcB is lower than SipZ, whereas SipY is dispensable for bacterial virulence.

In order to gain further insight into the possible differences in the specificity of these three SPases I at a global scale, the exoproteome was investigated here using single and double knock-out mutants of *L. monocytogenes*. Following a secretome-based approach, the exoproteomes of *L. monocytogenes* wild type (*wt*), *sipX*$^-$, *sipY*$^-$, *sipZ*$^-$, Δ*sipXY* and Δ*sipYZ* were resolved by 2-DE and further compared to determine the differential role of the multiple SPases I in the secretome.

2. Material and methods

2.1. Bacterial strains

As previously described [33], bacterial cells were grown in chemically defined medium MCDB 202 at 37 °C under shaking at an initial $OD_{600\ nm}$ of 0.01. Besides *L. monocytogenes* EGD-e wild type (*wt*) [36], five isogenic strains mutated in the multiple SPases I were investigated, *i.e. L. monocytogenes sipX*$^-$, *sipY*$^-$, *sipZ*$^-$, Δ*sipXY* and Δ*sipYZ* [34].

Sampling of extracellular proteins

As previously described [37,38], bacterial cultures were sampled at early stationary phase at the same $OD_{600\ nm}$ and extracellular proteins were recovered from the culture supernatant. Briefly, supernatant was collected after centrifugation