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Proteomic characterization of the *Legionella micdadei* membranes

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INTRODUCTION & AIM

Legionella species are natural parasites of free-living protozoa that reside in lakes, ponds, or streams, where they feed on bacteria in biofilms. When protozoa engulf *Legionella* bacteria, these bacteria can evade intracellular degradation and proliferate within a replication-permissive compartment known as the *Legionella*-containing vacuole (LCV). The presence of both *Legionella* rods and a suitable amoeba host in the pipes and tanks of potable water systems is indispensable for the formation of aerosols, which can lead to human infections. *L. micdadei* is a human pathogen distributed worldwide, causing Legionnaires' disease, a severe form of pneumonia and respiratory tract infection. Pneumonia caused by *L. micdadei* is mainly associated with hospital infections and primarily affects immunosuppressed patients **[1]**.

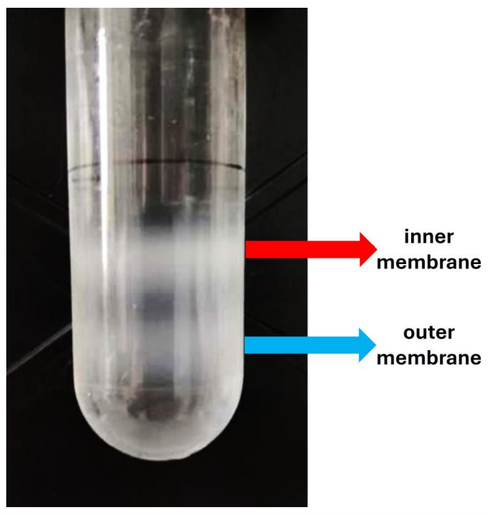
In bacterial pathogenicity, key factors include exported proteins that directly interact with host cells. *L. micdadei* possesses Dot/Icm type IVB and Lsp type II secretion systems, which serve to transport protein effectors and toxins into the extracellular environment or directly into the host cell, modifying host physiology and facilitating interactions with infected cells. Sequences of several classes of virulence factors have been identified in the *L. micdadei* genome, including Hsp60, type IV pili, macrophage infectivity potentiator (Mip), ferrous iron transport, alternative sigma factor RpoS, carbon storage regulator A, LetA/LetS two component, and RelA protein. Key virulence factors, such as proteins involved in oxygen binding, iron storage, transport across the host membrane, and some Dot/Icm substrates, are specific features of disease-causing pathogens **[2]**.

The study aimed to analyze the composition of the outer (OM) and inner membrane (IM) proteins isolated from *L. micdadei* bacteria.

METHODS

The bacterial mass collected from BCYE (Buffered Charcoal Yeast Extract) plates was digested with DNase and RNase and then disintegrated in a French press (SLM–Amico Instruments, Thermo Spectronic, Rochester, NY, USA) at 18,000 lb/in^2. Unlysed cells were removed by centrifugation for 20 min, 4°C, 1,000 x g (Sigma 6–16K).

A seven-step sucrose density gradient was employed to isolate the OM and IM of *L. micdadei* **(Fig. 1)**. The gradient, loaded with the sample, was subjected to ultracentrifugation at 114,000 × g for 20 hours at 4°C (Beckman Coulter, Brea, CA, USA). Membrane fractions were pelleted by ultracentrifugation for 60 min, 4°C, 100,000 x g (Beckman Coulter, Brea, CA, USA). The efficiency of membrane separation was verified by assessing protein content and membrane-specific enzyme markers in each fraction collected from the gradient. NADH oxidase activity, serving as an IM marker, and esterase activity, indicative of OM, were measured. The protein content of the OM and IM fractions was quantified using the Pierce™ BCA Protein Assay Kit.



Proteins isolated from the OM and IM membrane of *L. micdadei*, after prior digestion with trypsin, were analyzed using Liquid Chromatography with tandem mass spectrometry (LC-MS-MS). Proteins were divided into groups based on their functions or enzymatic activities using the UniProt, InterPro, KEGG, and Panther databases. The cellular localization of bacterial proteins was determined using the PSORTb v3.0 program.

Figure 1. *An example of OM and IM separation in a sucrose concentration gradient.*

RESULTS

A detailed analysis of the *L. micdadei* proteome revealed the presence of 1,748 proteins located in the OM and IM. The proteins identified in *L. micdadei* cell membranes included proteins involved in:

1) the transport of substances across cell membranes,
2) the synthesis of cell membrane components,
3) the ability to move,
4) the processing and transmission of environmental signals,
5) the response to stress conditions and in proteolysis,
6) in cell division,
7) the processing of genetic information,
8) the metabolic pathways of bacteria (energy production processes, metabolic pathways of carbohydrates, amino acids, lipids, fatty acids and nucleotides),
9) virulence factors.

The membranes of *L. micdadei* contained virulence factors, including proteins associated with the type IVB and type II secretion systems, as well as key virulence determinants such as the Major Outer Membrane Protein (MOMP), Mip, and EnhC. Additionally, *L. micdadei* harbored 20 proteins comprising the type IVB Dot/Icm secretion apparatus, including the lipoproteins DotC and DotD, along with DotA, DotB, DotF, DotH, DotI, DotK, DotL, DotM, DotN, DotO, DotP, IcmQ, IcmS, IcmT, IcmV, IcmW, IcmX, and LvgA **(Fig. 2)**.

The identified components of the *L. micdadei* type II secretion system (T2SS) included subunits T2SD, T2SE, T2SG, T2SL, and T2SM, which are integral to the secretory apparatus. Additionally, proteins likely constituting the T2SS secretome, exhibiting phospholipase and lysophospholipase activity, were detected.

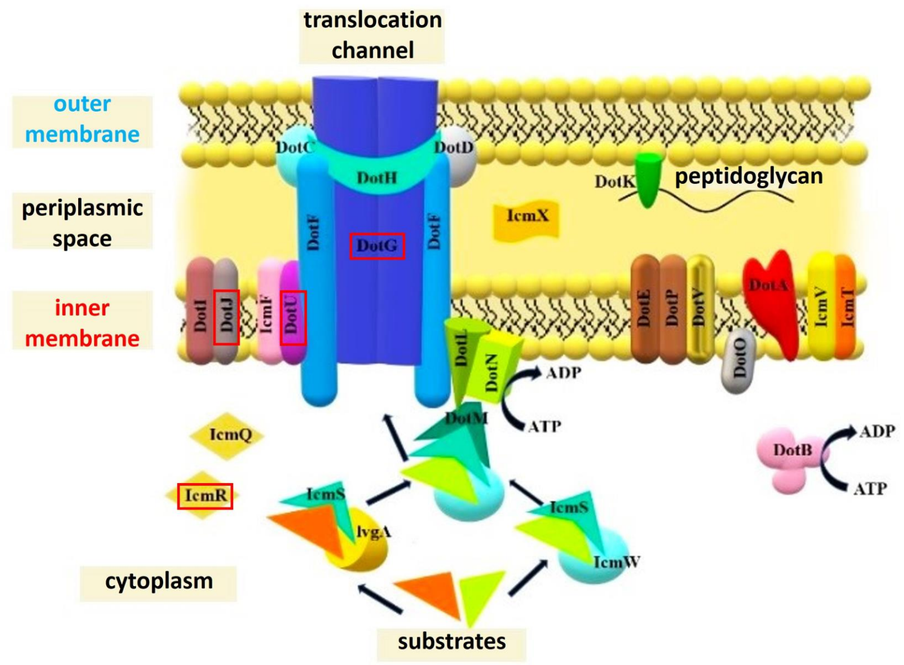


Figure 2. *Structure of the Dot/Icm secretion system of L. micdadei. Protein components of the Dot/Icm secretion system absent in the L. micdadei apparatus but present in the best described L. pneumophila type IVB secretion system are indicated in red boxes.*

Fractions of the *L. micdadei* cell membrane also contained LssZ proteins and a member of the HlyD family, both of which are associated with the bacterial type I secretion system (T1SS). Furthermore, proteins of the Tol-Pal system, which play a crucial role in bacterial cell division, were identified in the *L. micdadei* membranes. Several components spanning both the IM and OM were detected, including TolA, TolB, TolQ, and TolR, as well as the Pal lipoprotein.

Tol proteins are essential for the survival and pathogenicity of numerous bacterial pathogens. Notably, TolB has been investigated as a potential target for the development of a potent and highly immunogenic vaccine candidate against *L. pneumophila*.

Among the *L. micdadei* components involved in protein transport, several elements of the Sec translocon were identified, including the SecYEG complex, the SecA and SecD proteins, which are associated with the IM, and the SecF protein. Additionally, *L. micdadei* cell membranes contained proteins responsible for the proper localization of OM components. This group included proteins involved in the translocation of lipopolysaccharides (MsbA, LptB, LptD, and LptE) and lipoproteins (LolA, LolC, and LolD).

Another group of detected membrane-associated proteins comprised those involved in cell membrane and peptidoglycan synthesis, including cytoplasmic enzymes GlmM, GlmS, and GlmU, as well as MurA, MurB, MurC, MurD, MurE, MurF, MurI, MurJ, and MraY. Additionally, glycosyltransferases and transpeptidases were identified. The *L. micdadei* cell membranes also contained structural and functional components of the flagellum, including FliM, FliN, and FliO, as well as MotA and MotB, which are responsible for flagellar rotation.

Furthermore, elements of the two-component signal transduction system were detected, specifically histidine kinases (e.g., QseC) and response regulators.

Among the 1,784 identified proteins, 29 exhibited differential localization between the outer (OM) and inner (IM) membranes of *L. micdadei* **(Tab. 1)**.

Tabele 1. *Differences in the distribution of selected proteins in the OM and IM. (+) presence of protein; (-) absence of protein, (tr) trace amount.*

Identified proteins	IM	OM	Identified proteins	IM	OM
protein containing an iron-sulfur center	+	-	aldehyde activating protein	+	-
aspartyl protease	-	+	protein containing a DnaA domain	-	+
protein from the LicD family	tr	+	LysR family transcription regulator	+	-
diacylglycerol phosphatidylglycerol transferase - prolipoprotein	+	-	erythrose–4–phosphate dehydrogenase	+	-
CoA^+ binding protein	+	tr	amino acid permease	tr	+
EmrA protein	tr	+	D,D–transpeptidase MrdA	+	+
aminoglycoside phosphotransferase	+	tr	histidine kinase	+	+
FliN protein	-	+	ATP-binding ABC transporter	tr	tr

CONCLUSION

Proteomic analysis of *L. micdadei* membranes enabled the identification of proteins involved in various processes crucial for the proper functioning of the bacteria, including their survival, host cell penetration, and replication. The characterization of *L. micdadei* membrane proteins and the elucidation of their functions offer valuable insights into the mechanisms underlying the pathogenesis of these microorganisms. These findings contribute to a deeper understanding of the role of *L. micdadei* in host-pathogen interactions and its clinical relevance, potentially informing the development of targeted therapeutic and preventive strategies.

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