# Morphological and molecular characterization of cell wall-deficient L-forms of *L. monocytogenes*

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L-forms are cell wall-deficient forms of bacteria (CWDB). They represent bacterial variants which have lost the ability to maintain a rigid cell wall, and are thought to result from an induction process interfering with cell wall synthesis. Although several reports exist on morphological, serological and biochemical properties of L-forms, next to nothing is known about the molecular background for these unusual and interesting bacterial variants.

In this study several stable L-form lines were established from different *L. monocytogenes* strains. One of the L-form lines was established from strain Scott A constitutively expressing GFP. L-forms were isolated on media containing Penicillin G and foetal bovine serum, which could successively be substituted by milk serum.

Stabilization of the L-forms was performed by successive reduction of the antibiotic concentration in the culture medium. Eventually, L-forms were cultured in an osmotically stabilized soft agar medium without antibiotics. The different aspects of induction, growth and survival of L-forms were also investigated with respect to their growth in milk and confirmed that the milk matrix represents a suitable medium for L-form maintenance and growth.

Complete loss of the rigid cell wall was confirmed by confocal laser scanning microscopy and electron microscopy. Further characterization of the L-forms revealed that they can actively divide, and form both small, protoplast-like vesicles as well as multi-nucleated macrocells which appear to be surrounded by a single membrane.

Immunological analyses further confirmed the lack of a thick *Listeria*–type cell wall and specific cell wall-associated proteins such as Internalin A, whereas membrane-anchored proteins such as Internalin B were shown to be still present.

Based on time-lapse microscopy observations of GFP-labeled cells, a new hypothetical model for L-form growth and division is proposed. Replication by an "internal budding" process may explain their ability to multiply without a cell wall.

Transcriptome analysis and gene expression profiling was performed in order to compare global gene expression of parental *L. monocytogenes* Scott A and the respective L-forms. Whole genome macroarray analysis results, further validated by Real-Time PCR gene expression analysis, indicated manifold differences in gene expression and suggested that

specific genes are implicated in the different morphology and physiology of the two distinct variants. The L-forms apparently show a downregulated metabolism, which may result from adaptation to their increased volume and growth rate. Furthermore, the lack of a rigid cell wall results in a strong up-regulation of stress-related-genes. Although an intact cell wall is not present, the respective genes were shown to be expressed by the L-forms.

The potential pathogenicity of *L. monocytogenes* L-forms in cell invasion assays was studied *in vitro* by fluorescence microscopy and time-lapse confocal laser scanning microscopy. The results suggested that, under specific conditions, *Listeria* L-forms may intracellularly survive and persist after engulfment by macrophages. This observation supports the hypothesis that *L. monocytogenes* L-forms may represent stealth pathogens able to escape from host immune response.

The presented work provided the basis for an extensive characterization process leading to an improved understanding of the L-form cell and the significance of the transformation for the potential infection of eukaryotic cells. The occurrence of L-form cells in many bacterial species, together with the results from this study; clearly disprove the opinion that L-form cells only represent artefacts during the presence of cell-wall active antibiotics. The transformation to a cell wall deficient L-form may represent an advantageous strategy for bacteria in order to escape from environmental conditions that have a detrimental effect on cell wall-bearing bacteria, like antibiotics, bacteriophages or host immune response.

In conclusion, the presented results from physiological, morphological and molecular investigations demonstrate that stable *L. monocytogenes* L-forms represent viable bacteria that were adapted to their novel cellular conformation. They are not only able to survive but also have the ability to replicate, grow and persist as stealth pathogens within eukaryotic cells.