Advances in understanding the molecular basis of pathogenesis of *Listeria monocytogenes*

Werner Goebel
Biocenter, University of Würzburg
In the old days

...and some of her historic highlights
How it all began:

H. Seeliger (1921-1997)
Professor of Hygiene and Microbiology at the University of Würzburg 1965-1989
Phase I (1980-86)

- Uropathogenic *E. coli* – first virulence determinants (Hly and S- fimbriae genes)
- Chromosomal Pathogenicity (Hly)-Islands
- Cytolysins of Gram-positive bacteria
Phase II 1986-1991

Start of Research in *Listeria monocytogenes*
with T. Chakraborty, S. Kathariou, J. Kreft
- LLO as virulence factor (S.K.)
- PrfA and the PrfA-gene cluster (T.C)
- ActA and ActA-induced actin polymerization (T.C)
- LLO, ILO, SLO (J.K)
H. Seeliger ....

........at ISOPOL X, Pecs, Hungary, August 1988
Expression of most virulence genes depends on the transcriptional activator PrfA
Later work:

M. Kuhn:
- iap/p60
- Host cell responses
R. Böckmann, C. Dickneite, S. Bohne
- PrfA-dependent in vitro transcription
F. Engelbrecht
- Small internalins of Lm and Liv (inlC u.a.)
A. Bubert/M. Goetz/B. Joseph/J. Slaghuis/J. Schaer
- Metabolism and virulence
S. Altrock/Q. Luo/S. Mertins/R. Ecke
- PrfA activity and carbohydrate metabolism
S. Pilgrim/J. Stritzker/C. Schoen/Loeffler
- Lm as carrier for DNA, RNA and protein
Links between metabolism and virulence

- Carbon (C)-metabolism under extra- and intracellular conditions (PrfA activity and C-metabolism)

*L. monocytogenes* as carrier system

- Transfer of functional DNA and RNA into tumor cells
- Specific targeting of *L. monocytogenes* to (and into) tumor cells
Part I

Links between Metabolism and Virulence in *L. monocytogenes*
The prfA mutant is highly virulence – attenuated

**Example 1:** A pycA mutant (defective in pyruvate carboxylase) is as avirulent as the prfA mutant

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<thead>
<tr>
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<th>EGDe</th>
<th>prfA</th>
<th>pycA</th>
<th>prfA</th>
<th>pycA</th>
<th>prfA</th>
<th>pycA</th>
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<tr>
<td>CFU log10 at day 3 post infection</td>
<td>5x10³</td>
<td>5x10³</td>
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Example 2: InlA production and internalization (into Caco-2 cells) of an *aroA* mutant is highly enhanced.
Example 3: PrfA activity – and hence *L. monocytogenes* virulence – is intimately linked to the metabolic conditions

-PrfA activity is inhibited in presence of PTS-sugars, like cellobiose, glucose or mannose

-PrfA mutants (PrfA*) were isolated (J.A. Vazquez-Boland and co-workers; N. Freitag and co-workers) that are constitutively active, i.e. PrfA* activity is no longer modulated in presence of PTS sugars
The carbon (C) - metabolism of *L. monocytogenes* under extra- and intracellular conditions was determined by:

- Transcriptome analysis and
- $^{13}\text{C}$-Glucose Flux Measurement (by NMR or MS) in defined minimal media (with glucose or glycerol as carbon sources) and in J774 macrophages (in cooperation with the group of A. Bacher and W. Eisenreich, TU Munich)

*L. monocytogenes* and *L. innocua* genome sequence (Glaser et al. 2002)

Listeria genus genome sequence performed in collaboration between Institut Pasteur and the German „Pathogenomics“ network (unpublished)
Primary metabolism in presence of glucose

Active carbon catabolite repression control (CCR)

Glucose-6-P → Gluconolactone → Pentose-Phosphate-cycle

Glycolysis

Ser, Gly → eno → PEP → Pyruvate → Acetyl-CoA → Oxaloacetate

Asp, Thr, Lys, Cys, Met

Acetyl-CoA + CO2/ATP → Oxaloacetate → Succinyl-CoA → 2-Oxoglutarate → Glu, Arg, Pro

PrfA – low activity

HPr-His-P, HPr-Ser-P, HPr

CCR

Eisenreich et al. PNAS 2005; Ecke et al., unpublished
With C3-compounds (glycerol or dihydroxyacetone) as carbon sources

Growth in presence of glycerol leads to (partial) CCR relieve

Joseph et al., unpublished
Even when the cellular concentration of PrfA protein is as high as PrfA* protein PrfA activity is low in presence of glucose and cellobiose but high in presence of glycerol.
Glucose

PEP:PTS

Membrane

Enzyme II\textsubscript{Glc}

Glycerol

GlpF

Inactive PrfA

Active PrfA
Analysis of interactions of purified listerial proteins involved in PTS/CCR control with PrfA

(protein-protein-interactions measured by BIACORE )

HPr and HPr-Ser-P do not interact with PrfA
In vitro transcription
Influence of HPr, HPr-SerP and EIIA on PrfA-activity

- No inhibition of PrfA-activity by HPr or HPr-Ser46P
- Weak inhibition of PrfA-activity by EIIA
Deduced intracellular C-metabolism of *L. monocytogenes* (in J774 macrophages)

Glycerol or other C3-C source

Glycerol-3-P

GAP ↔ Dha-P

F1-6DP

Glu-6-P

Gluconolactone-6P

- CO₂

PPP

High PrfA activity:
-expression of all PrfA-dependent genes is induced

GlpK

GlpD

Serine, Glycine

PEP

Valine

Pyruvate

Alanine

Acetyl-CoA

+ CO₂, ATP

Pyk

Asparate

Oxaloacetate

Citrate

Oxoglutarate

Glutamate

Eilert et al. unpublished
Glycerol

GlpF1 (lmo1539)

Glycerol

GlpF2 (lmo1167)

Glycerol

Dha

Gly-3-P

Gluc-6-P

G-3-P

DHAP

GAP

Joseph et al., unpublished

Glucose-6-P

GAP

DhaP

Dihydroxyacetone
A mutant unable to perform C3 metabolism, i.e. deficient in
- the two *glpK* (glycerol-kinase) genes,
- *glpD* (glycerol-3-P dehydrogenase), and
- the two *dhaK* genes

show a strongly reduced intracellular replication efficiency
in macrophage- and epithelial cells under the *in vitro* culture conditions.
The *pycA* mutant is also unable to replicate in Caco-2 cells.

![Graph showing intracellular bacterial counts over time](image)

Intracellular bacteria

- Wild-type strain
- *pycA* mutant

**Time of intracellular replication in Caco-2 cells:**

- 1, 3, 5, 7, 9 hours post-infection (h pi)

**Log10 scale:**

- 10^5, 10^6, 10^7

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**Viable bacterial counts log10**: This refers to the logarithmic scale used to represent bacterial counts in the graph.
Enhanced invasion and InlA production of the *aroA* mutant

Stritzker et al., 2005
The *aroA* mutant is unable to produce aromatic amino acids but also menaquinone which is the only quinone of Lm (required for aerobic respiration).

- Due to the inability to produce menaquinone the *aroA* mutant performs, like a *menF* mutant, a predominantly anaerobic metabolism,
The anaerobic metabolism leads to

- slight activation of PrfA and to

- strong activation of the translation of inlA(B) mRNA
  (possibly by a riboswitch mechanism of inlA(B) mRNA under anaerobic conditions)

-Due to the anaerobic metabolism intracellular replication of the aroA mutant is strongly impaired and hence the virulence of this mutant is highly attenuated (LD50 > 10^7)

- this mutant is an excellent carrier for the introduction of protein, RNA and DNA into mammalian cells
Conclusion I

- *L. monocytogenes* can use various PTS carbohydrates as well as glycerol as carbon sources for extracellular growth.

- Uptake of PTS carbohydrates (causing CCR) inhibits PrfA activity (cellobiose>glucose> mannose), whereas that of glycerol (partially relieving CCR) leads to high PrfA activity.

- However, the two major components of the CCR control, CcpA and Hpr-Ser-P, are not direct modulators of PrfA activity.

- C3 carbon sources seem to play also a major role for intracellular growth of *L. monocytogenes* (at least in *in vitro* cell cultures).

- Pyruvate carboxylase (PycA) is an essential enzyme for extra- and intracellular listerial growth and hence a good metabolic target for the screening of antilisterial drugs.

- An *aroA* mutant performs anaerobic metabolism, is highly virulence attenuated but shows increased InlA-mediated invasiveness due to increased production of InlA (riboswitch of inlA(B) mRNA under anaerobic conditions ?)
Part II

*Listeria monocytogenes* as carrier for release of functional DNA-, RNA- and protein into mammalian cells
Three approaches for delivering protein or protein-encoding DNA or RNA into mammalian cells via *L. monocytogenes*:

a. By production and secretion of a protein-antigen by the carrier bacteria after uptake by APC (DC and MP)

Lm as bacterial carrier with the gene (__) for an antigen

Host cell

b. By delivery of DNA und RNA, encoding such antigens oder other functional proteins into the cytosol of APC and other host cells which express the genetic information

DNA mRNA

Phage lysin-mediated autolysis of the bacterial carrier

Plasmid-DNA mRNA (with 5'-IRES-element)
Delivery of DNA and mRNA encoding EGFP by autolysing *Listeria monocytogenes* works equally well in epithelial cells (with bactofection rates up to 10%) 

Pilgrim et al. 2003; Schoen et al., 2005
Gene directed enzyme prodrug therapy (GDEPT)

Delivery of enzymes for conversion of non-toxic prodrugs into cell toxic drugs by using autolysing aro strains
Used Prodrug-Drug-Systems

- Conversion of **6-Methylpurine deoxyribose (MePdR)** into the cell toxic drug **6-Methylpurine (MeP)** by PNP

  ![Diagram](prodrug-drug-system-1.png)

  \[\text{ProDrug} \xrightarrow{\text{MePdR}} \text{PNP} \xrightarrow{\text{MeP}} \text{Drug}\]

  **deoD** encodes *E. coli* purine nucleoside phosphorylase (PNP)

- Conversion of **5-Fluorocytosine (5-FC)** into cell toxic drug **5-Fluorouridine- monophosphate (5-FUMP)** by CDA/UPT the gene product of fused gene *fcul*

  ![Diagram](prodrug-drug-system-2.png)

  \[\text{ProDrug} \xrightarrow{\text{5-FC}} \text{CDA} \xrightarrow{\text{5-FU}} \text{UPT} \xrightarrow{\text{5-FUMP}} \text{Drug}\]

  **fcul**: gene fusion of yeast encoding cytosine deaminase (CDA) and uracil phosphoribosyltransferase (UPT)
Melanoma B16 cells are killed upon *L. monocytogenes* – mediated transfer of genes encoding prodrug-drug converting enzymes after treatment with the prodrugs
Luciferase-expressing *Listeria monocytogenes* as carriers of cell-toxic compounds into tumor tissue

Infected with *Lm* without DNA for Prodrug-Drug converting Enzyme

Infected with *Lm* with DNA for Prodrug-Drug converting enzyme

Tag 14

ca $10^5$ bacteria within the tumor

Rel. enzyme activity after 14 days

30

60
Targeting of *L. monocytogenes* to specific tumor cells
Internalization of *L. monocytogenes* into mammalian cells occurs by a zipper mechanism

1. *L. monocytogenes* is equipped with a receptor-specific ligand L (InlA or InlB)

Mammalian cell with receptor R (E-cadherin or Met)
Targeting of *L. monocytogenes* to specific cells

2. *L. monocytogenes* equipped with receptor-specific ligand L binds to receptor R

Mammalian cell with specific receptor R
Targeting of *L. monocytogenes* to specific cells

3. *L. monocytogenes* is taken up by receptor – ligand interaction

Mammalian cell with specific receptor R
Targeting of *L. monocytogenes* to specific tumor cell

1. *L. monocytogenes* equipped with a receptor-specific ligand L (antibody against tumor receptor)

Tumor cell with over-expressed specific receptor, e.g. Her2/neu
Targeting of *L. monocytogenes* to specific tumor cell

2. *L. monocytogenes* equipped with antibody against tumor receptor – specific recognition?

Tumor cell with over-expressed specific receptor, e.g. Her2/neu
Targeting of *L. monocytogenes* to specific tumor cell

3. *L. monocytogenes* equipped antibody against tumor receptor - specific internalization ??

Tumor cell with over-expressed specific receptor, e.g. Her2/neu
Expression of *S. aureus* protein A (SPA) on the surface of *L. monocytogenes* (Lm-spa+) and binding of FITS-labelleld antibodies to the listerial surface
Specific invasion of *Lm inlAB, spa+*, loaded with Herceptin® (monoclonal antibody against Her2/neu), into Her2/neu - overexpressing cancer cells.

SKBR-3 cells (Her2/neu +++)

Caco-2 cells (Her2/neu -)

HepG-2 (Her2/neu +)
This new type of antibody/receptor-mediated internalization of *Listeria monocytogenes* into tumor cells is not observed with unspecific antibodies.
Herceptin®/HER-2/neu-mediated invasion of Lm-spa⁺ is followed by escape of Lm-spa⁺ into the cytosol of the tumor cells and efficient replication
a. Lm-PactA-gfp (WT control)

b. Lm inlA/B, spa+--PactA-gfp

c. Lm aroA,inlA/B--PactA-gfp

d. Lm aroA,inlA/B,spa+-PactA-gfp

SK-BR-3 cells

1h 6h 24h

without

with Herceptin®

without

with Herceptin®

without

with Herceptin®

without

with Herceptin®
Conclusions II

- Efficient transfer of DNA and RNA encoding functional proteins into mammalian cells including primary phagocytes ("bactofection"), can be achieved by the virulence-attenuated aroA L. monocytogenes mutant.

- Release of DNA and RNA is greatly enhanced by phage lysin-mediated intracellular destruction of the carrier bacteria.

- RNA delivery results in a considerably earlier expression of the gene product in the bactofected cells (including APC) than DNA delivery and leads to a more efficient cellular immune response (mainly CTL).

- Specific targeting of L. monocytogenes to tumor cells can be achieved by receptor-antibody interaction and may improve the specific delivery of anti-cancerogenic agents into tumor cells.
Who did the work:

- Susanne Bauer
- Regine Ecke
- Alexa Frentzen
- Monika Goetz
- Biju Joseph
- Karin Knuth
- Sonja Mertins
- Steffi Müller-Altrock
- Daniela Loeffler
- Sabine Pilgrim
- Jenny Schaer
- Christoph Schoen
- Jörg Slaghuis
- Jochen Stritzker
Collaborations

Institut für Lebensmittelchemie, Würzburg:
Marcus Taupp
Peter Schreier

Institut für Medizinische Strahlenkunde und Zellforschung, Würzburg:
Joachim Fensterle
Ivaylo Gentschev
Ulf R. Rapp

Lehrstuhl für Physiologische Chemie II, Würzburg:
Ernst Conzelmann

Virchow Center, Würzburg:
A. Szalay

Institut für Biochemie TU München
Adalbert Bacher/Wolfgang Eisenreich

Department of Veterinarian Medicine, University of Bristol
J.A. Vazquez-Boland
Thank you all!

and

......all the best for your research on *Listeria monocytogenes*!
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- A. Szalay

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- Marcus Taupp
- Peter Schreier

Institut für Biochemie TU München
- Adalbert Bacher/Wolfgang Eisenreich

Department of Veterinarian Medicine, University of Bristol
- J.A. Vazquez-Boland
Glucose-6-phosphate

- **Other PTS**
  - P
- **PTS-Gluc/Man**
  - (Lmo0096-98; lmo0781)
  - P

**Glycolysis**
- Ser, Gly
- Ala (Val, Ile, Leu)
- Asp, Thr, Lys, Cys, Met
- + CO₂

- **Glucose-6-P**
  - Glucose-6-phosphate
  - hpt

**PEP**
- Pyruvate
  - pycA
  - pyk
  - pdh

**Acetyl-CoA**
- Oxaloacetate
- Succinyl-CoA
- 2-Oxoglutarate
- Glu, Arg, Pro

**Gluconolactone**
- eno
- -CO₂

**Pyruvate**
- Xylulose-5-P
  - Erythrose-4-P
  - Tyr, Phe, Trp
  - Heptulose-7-P

**Pentose-Phosphate-Cycle**
- CcpA
- CCR

**Citrate cycle**
- PrfA – low activity
- PrfA – high activity

**HPr**
- HPr-Ser-P
- HPr-His-P
- hprK

**HPr-His-P**

**Eisenreich et al. PNAS 2005; Ecke et al., unpublished**
Oral infection, i.v. or i.p. injection into the vaccinated organism

Transformation into the carrier strain *L. monocytogenes ΔtrpS*

**DNA Delivery Plasmid pSP118**

- **Plasmid pSP18**
  - **origin of replication for** *L. monocytogenes*
  - **gene for** trpS
  - **gene for phage lysin (ply118) under PactA control**
  - **origin of replication for** *E. coli*
  - **eukaryotic promoter**
  - **cDNA of the antigen**
  - **polyadenylation signal**

*Oral infection, i.v. or i.p. injection into the vaccinated organism*
Delivery of plasmid DNA encoding EGFP to different cell lines – DNA expressed by the host cell after being released from the lysed bacteria

Problem: EGFP expression is slow and occurs only 24 – 48 post bactofection
Components of listerial CCR that are apparently not involved in modulation of PrfA activity

PEP:PTS

Glucose

Enzyme II$^{\text{Glc}}$

IIA$^{\text{Glc}}$

IIB$^{\text{Glc}}$

IIIC$^{\text{Glc}}$

PEP:PTS

Glucose-6-P

HPr

H15-P
**L. monocytogenes** has a large number of PTS genes (for 29 complete PTS and 8 incomplete ones): among those at least 6 are involved in glucose transport and at least 8 in cellobiose transport.
HPr-His-P-mediated phosphorylation state and PrfA activity in presence of **celllobiose or glucose** (PTS sugars)

Active CCR leads to repression of:

**PEP:PTS**

Glucose and other PTS sugars

**Enzyme II**

**IIA**

**IIB**

**IIC**

Membrane

**PrfA**

**PRD-Regulators-P**

**HPr-His-P**

**Glycerol-kinase-P**

**Dha-P**

**Dha-K-P**

**PEP**

**HPr-His-P**-mediated phosphorylation state and PrfA activity in presence of **celllobiose or glucose** (PTS sugars)

**Glucose**

**celllobiose**

**mannose**

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<td><strong>OD_{600nm}</strong></td>
<td>G</td>
<td>G</td>
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<td><strong>incubation</strong></td>
<td>G</td>
<td>G</td>
<td>M</td>
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<tr>
<td><strong>HPr</strong></td>
<td>+</td>
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**HPr conjugates**

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**celllobiose, glucose, other PTS-sugars (under aerobic conditions)**

**different levels of Hpr-His-P**

**active CCR, different levels of**

**HPr-Ser-P**

**Low PrfA activity**
Many PTS expressed

Relieved CCR results in expression of:

- Many PTS expressed

Enzyme II

IIC

IIB

IIA

PEP:PTS

Membrane

PrfA

HPr-His-P

PRD-Regulators-P

Glycerol-kinase-P

DhaK-P

Dha-P

OD: 0.6 1.0 0.6 1.0 0.6 1.0

incubation (70°C): G G G C C C Y Y Y Y

HPr

HPr-His-P

HPr-Ser-P

Glycerol-kinase-P

G = glucose  C = cellobiose  Y = glycerol

High PrfA activity

High HPr-His-P

Relieved CCR, low HPr-Ser-P

glycerol

HPr-His-P-mediated phosphorylation state and PrfA activity in presence of glycerol
Passage of introduced DNA through the nucleus is slow: expression of antigen takes 24-48 h – during this time apoptosis of APC occurs.
Solution:
Delivery of mRNA by autolysing *Listeria monocytogenes* circumvents passage through the host cells nucleus.
Comparison of DNA, RNA and protein delivery efficiencies *in vivo*

**OT-I transfer**

*(CD8 response)*

11.3

**OT-II transfer**

*(CD4 response)*

0.6

0.4

L. *monocytogenes*-mediated transfer of antigen-encoding DNA or RNA results mainly in CD8 T cell response  
(Loeffler et al, 2006)