# Australian Research Council Centre of Excellence

## Structural and Functional Microbial Genomics

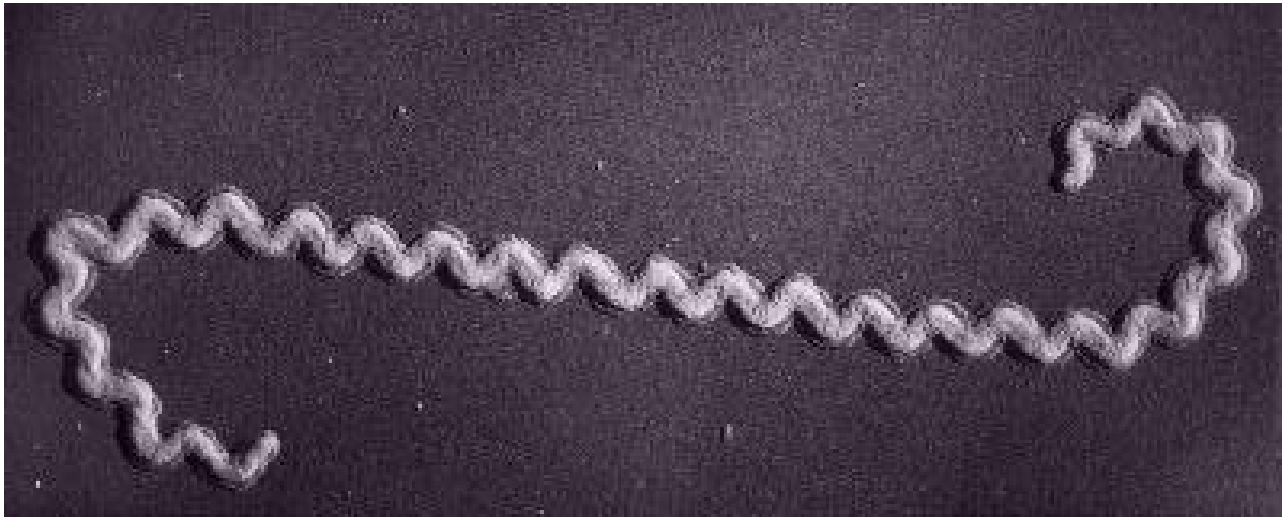
### Professor Ben Adler, Director

Microbial Genomics
Structure and Function

Australian Government
Australian Research Council

MONASH University

# *Leptospira*



Genomics
Pathogenesis
Vaccines

# Can genomics help us understand a bacterial pathogen?

**OR**

# What can we do with a difficult organism?

**OR**

# Why things sometimes don’t work out how we expected (wanted)

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# *Leptospira*

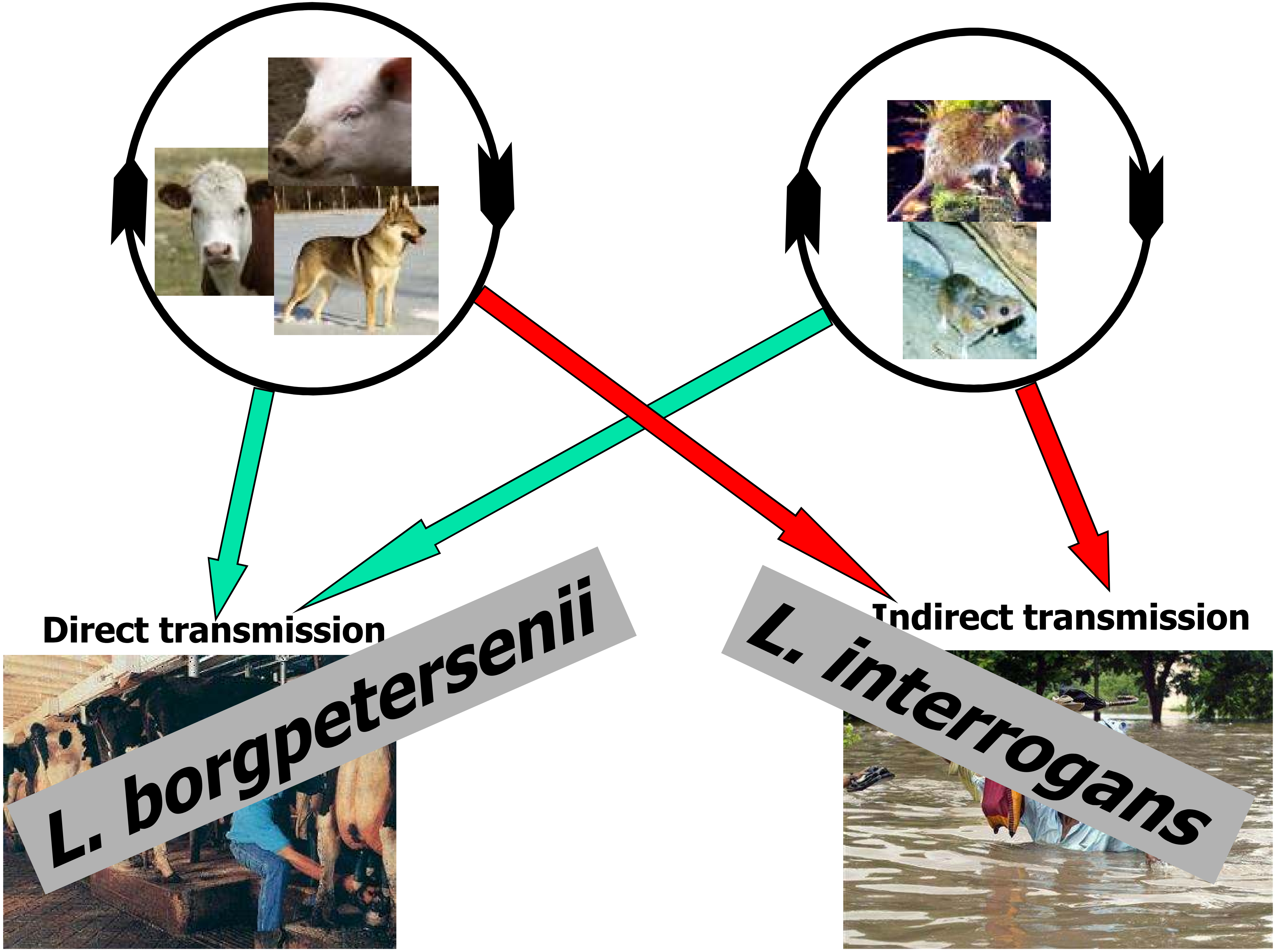
**Pathogens**

- ***L. interrogans, L. borgpetersenii*** **and at least 14 other species**
- **>200 serovars (e.g. Copenhageni*, Hardjo*)**
- **Most widespread zoonosis**
- **1M cases worldwide – up to 20% mortality**
- **Infections of cattle, swine, dogs,…**
- **Very few genetic systems available**

**Saprophytes**

- ***L. biflexa*** **and others**
- **>100 serovars (e.g. Patoc*)**

***first genome sequences**



Direct transmission

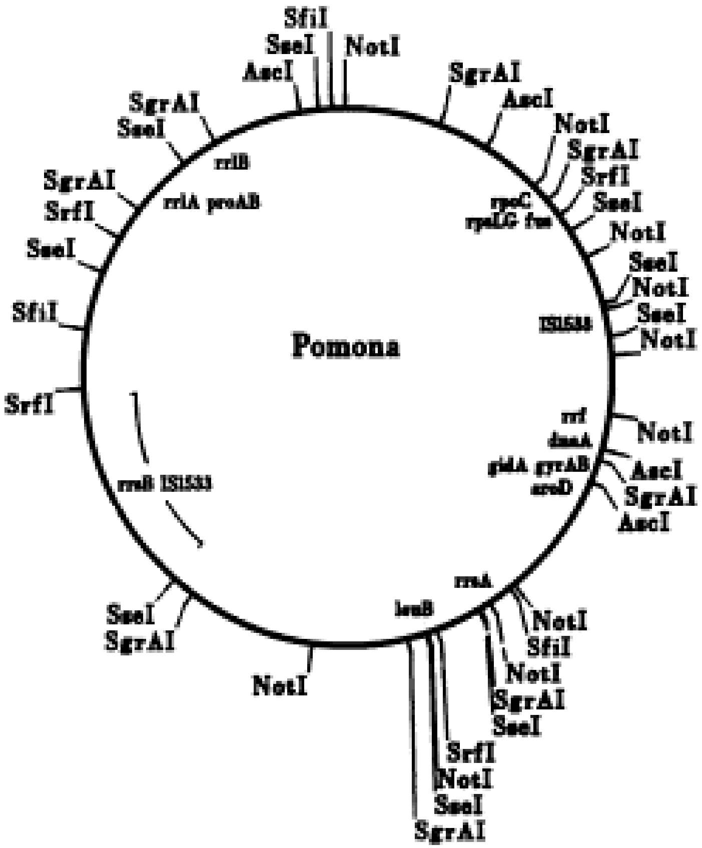
*L. borgpetersenii*

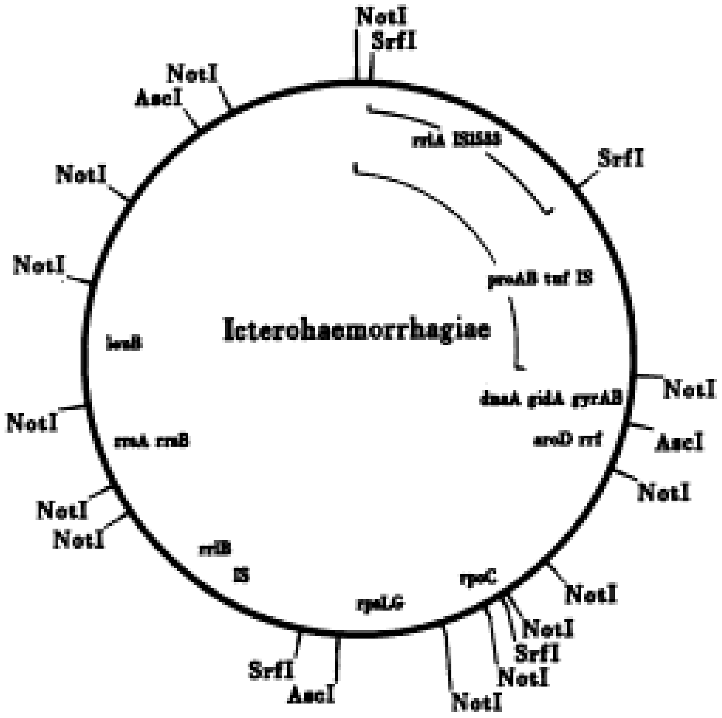
Indirect transmission

*L. interrogans*

# What did we have before genomics?

- Rough genome size and "map"





# What did we have before genomics?

- Rough genome size and “map”
- No plasmids
- No bacteriophages
- No transformation system
- No mutagenesis system
- Colonies take 4 weeks to grow (if you are lucky)

- i.e. **Nothing!**

# *L. interrogans* vs *L. borgpetersenii*

- *L. interrogans* (sv Copenhageni)
  - severe infection – Weil's disease
  - high mortality rate if untreated
  - rodent maintenance host
  - direct and indirect spread

- *L. borgpetersenii* (sv Hardjo)
  - much less severe infection
  - never fatal
  - bovine maintenance host
  - usually direct spread

- Half of all serovars (>90% of all cases) are in these two species

# Comparative genomics
## *L. interrogans* vs *L. borgpetersenii*

| | *L. interrogans* | *L. borgpetersenii* |
|---|---|---|
| No of genes | 3613 | 3453 |
| Size kb | 4691 | 3932 |
| % G+C | 36.7 | 41.3 |
| transposases/pseudogenes | 6% | $\boxed{20\%}$ |

# Pseudogenes in *L. borgpetersenii*

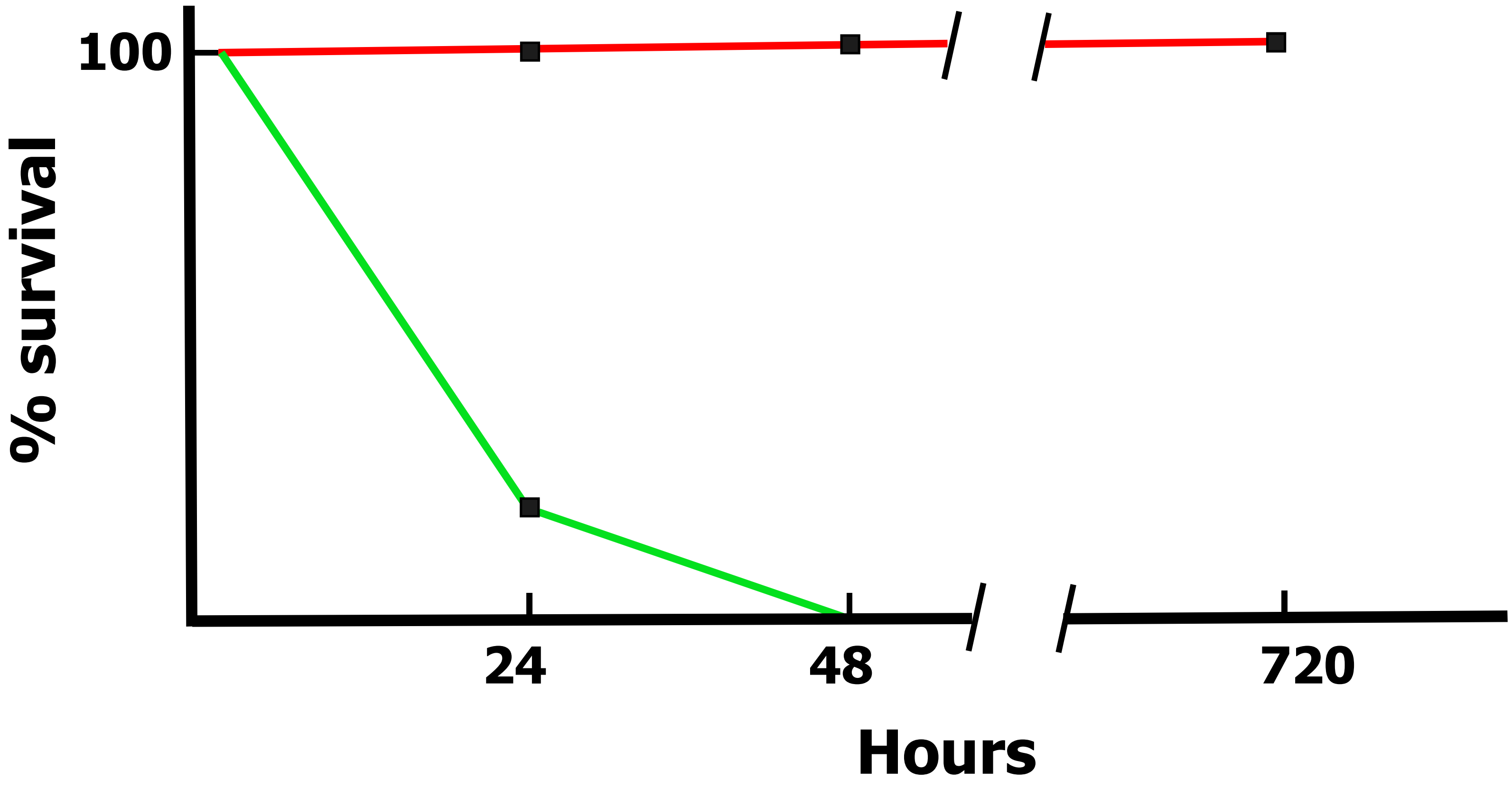
Pseudogenes in *L. borgpetersenii* not random

- environmental sensing
- nutrient transport
  - limits environmental adaptability
  - limits nutrient acquisition

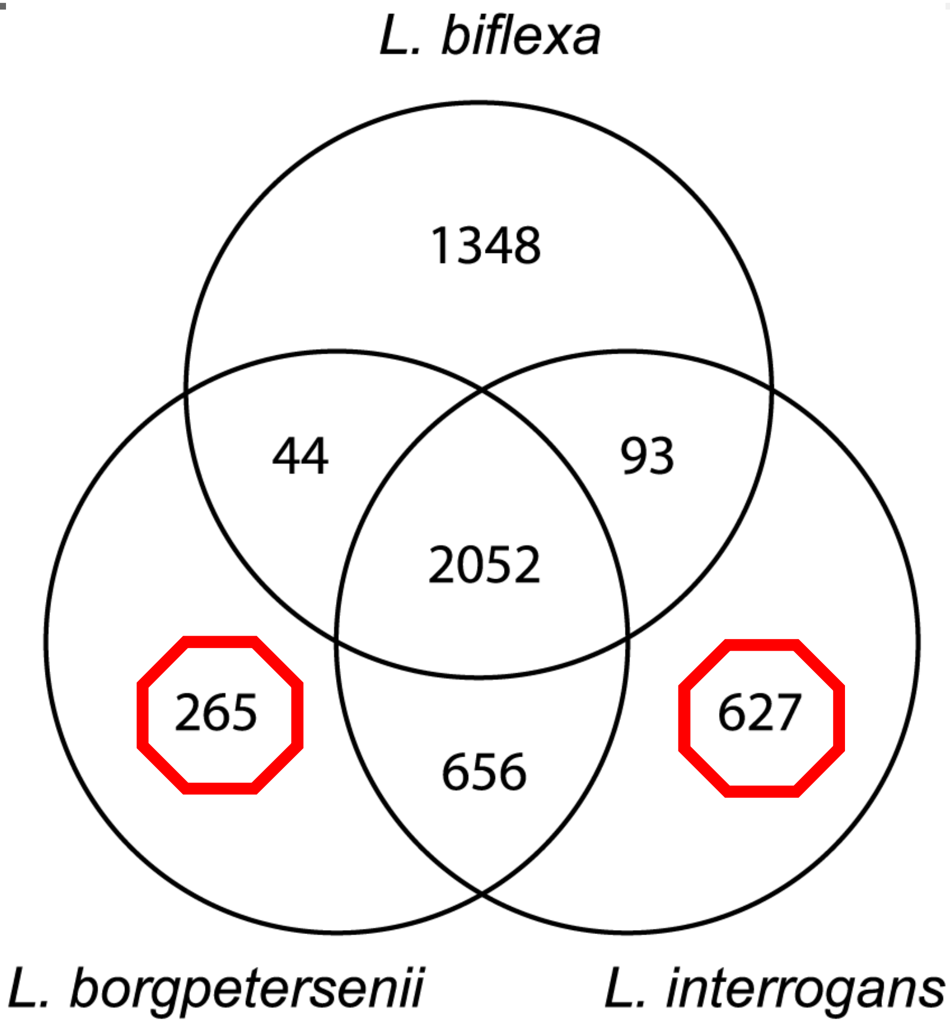
**What could this mean?**

- *L. borgpetersenii* is undergoing genome reduction
  - Genes no longer an advantage become non-functional
  - Reflect differences in transmission cycle and environment

# Survival in water
## *L. interrogans* vs *L. borgpetersenii*

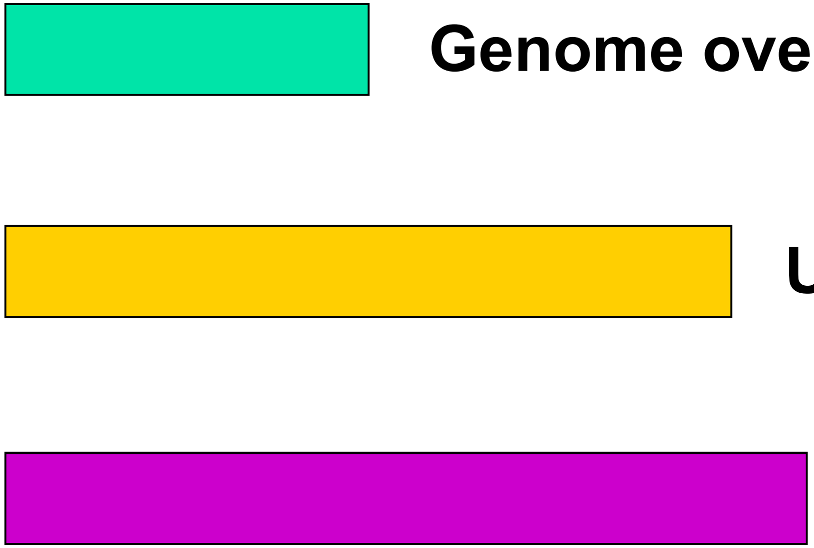


# Comparative genomics pathogens vs saprophyte



# Comparative genomics pathogen-specific genes

**% genes unknown function**



**Genome overall ~40%**

**Unique to *L. borgpetersenii* 76%**

**Unique to *L. interrogans* 82%**

**Genes of unknown function are over represented (so won’t tell us much about pathogenesis)**

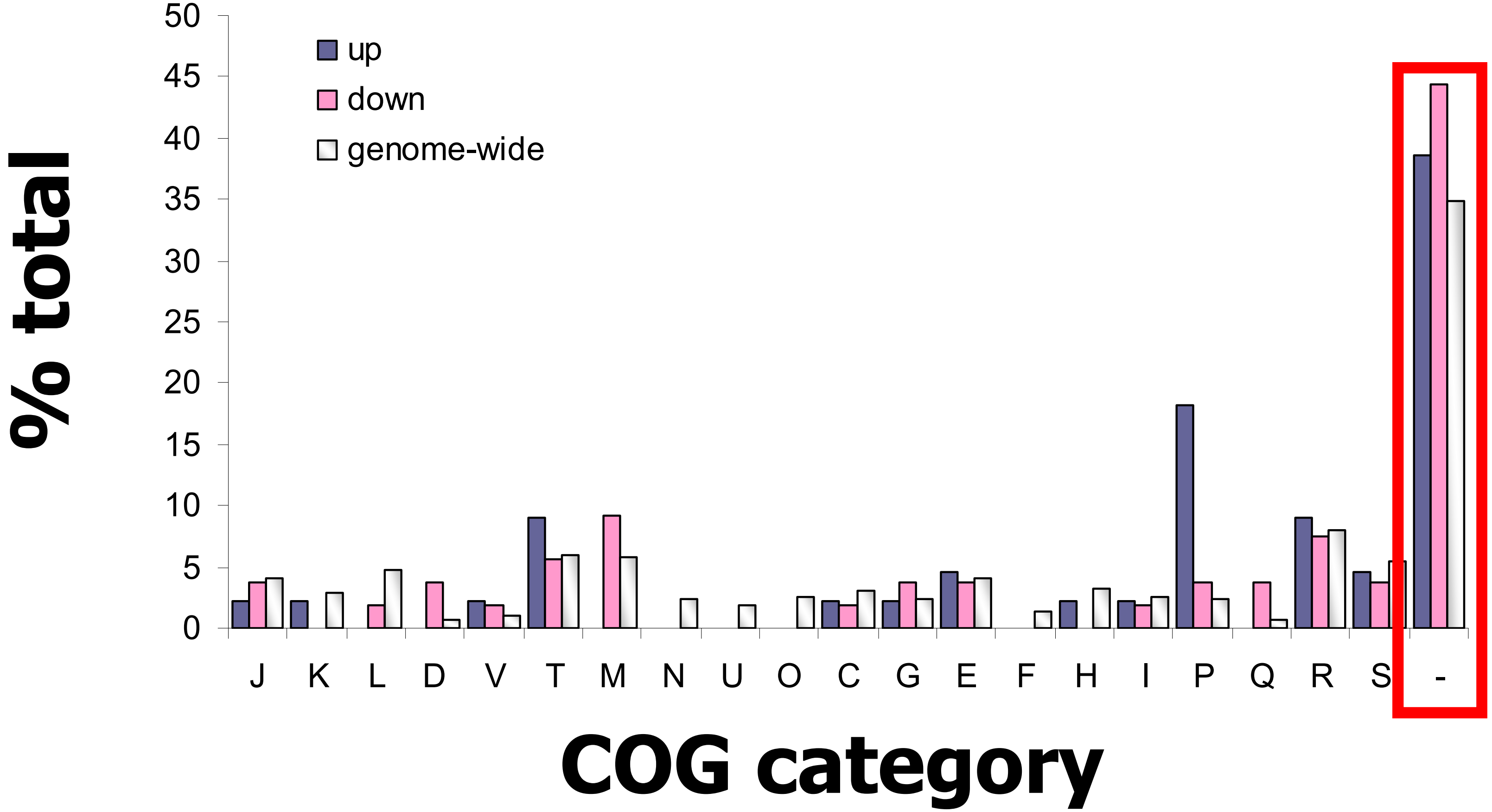
# What next?
# Genome-wide transcriptome studies

**Gene expression changes in response to:**

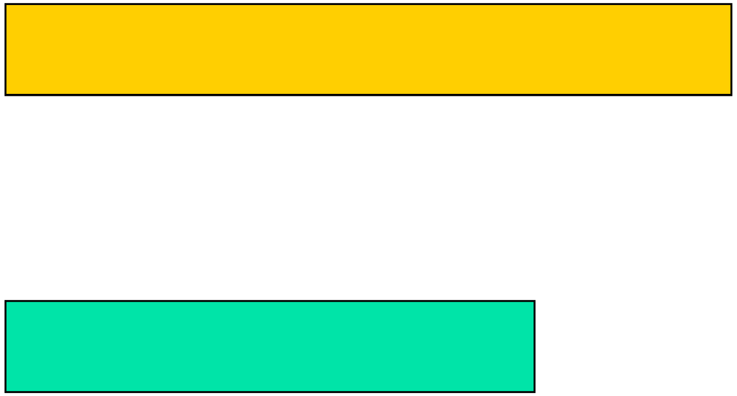
- Physiological/environmental temperature ~250 genes
- Physiological/environmental osmolarity ~220 genes
- Presence of serum ~55 genes
- Reduced iron concentration ~43 genes
- Implanted rat chambers ~166 genes

**Surely these genes will give us clues about pathogenesis!**

# Genes up/down regulated – low iron



# Genes up regulated – low iron

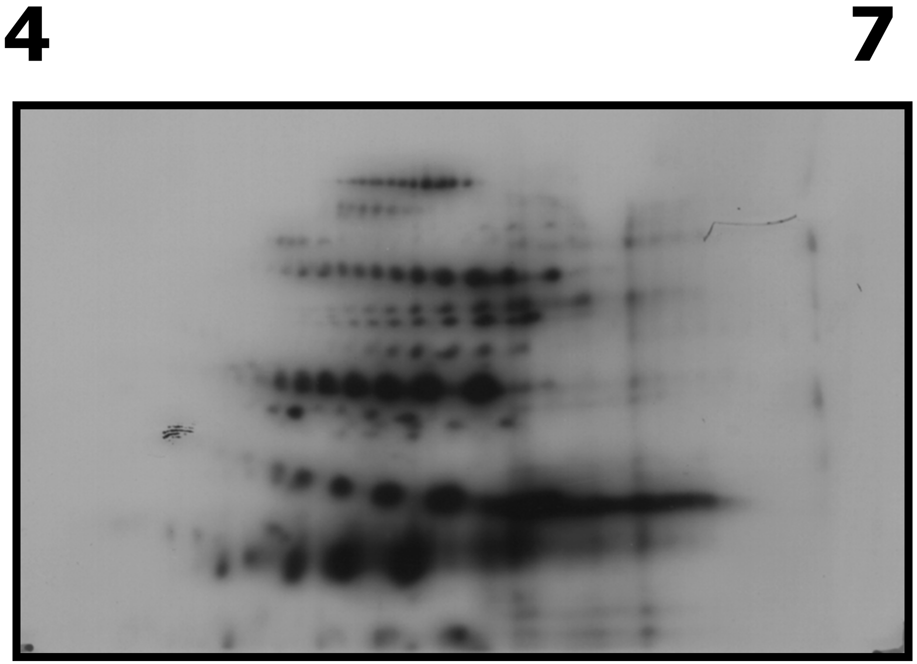


*L. Interrogans* unique 20 genes

Function unknown 16 genes

**What other approaches could be taken to identify these genes?**

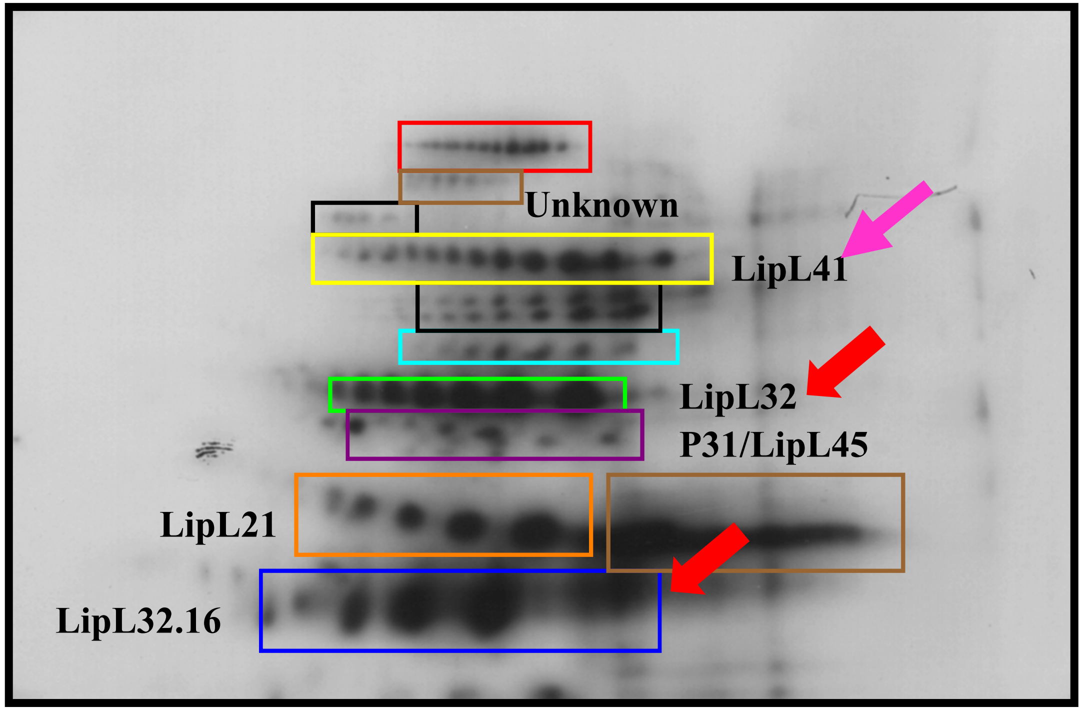
# Defining the leptospiral surface



**Biotin labeling of intact leptospires**

**Biotin labeling of leptospiral sonicate**

# Defining the leptospiral surface

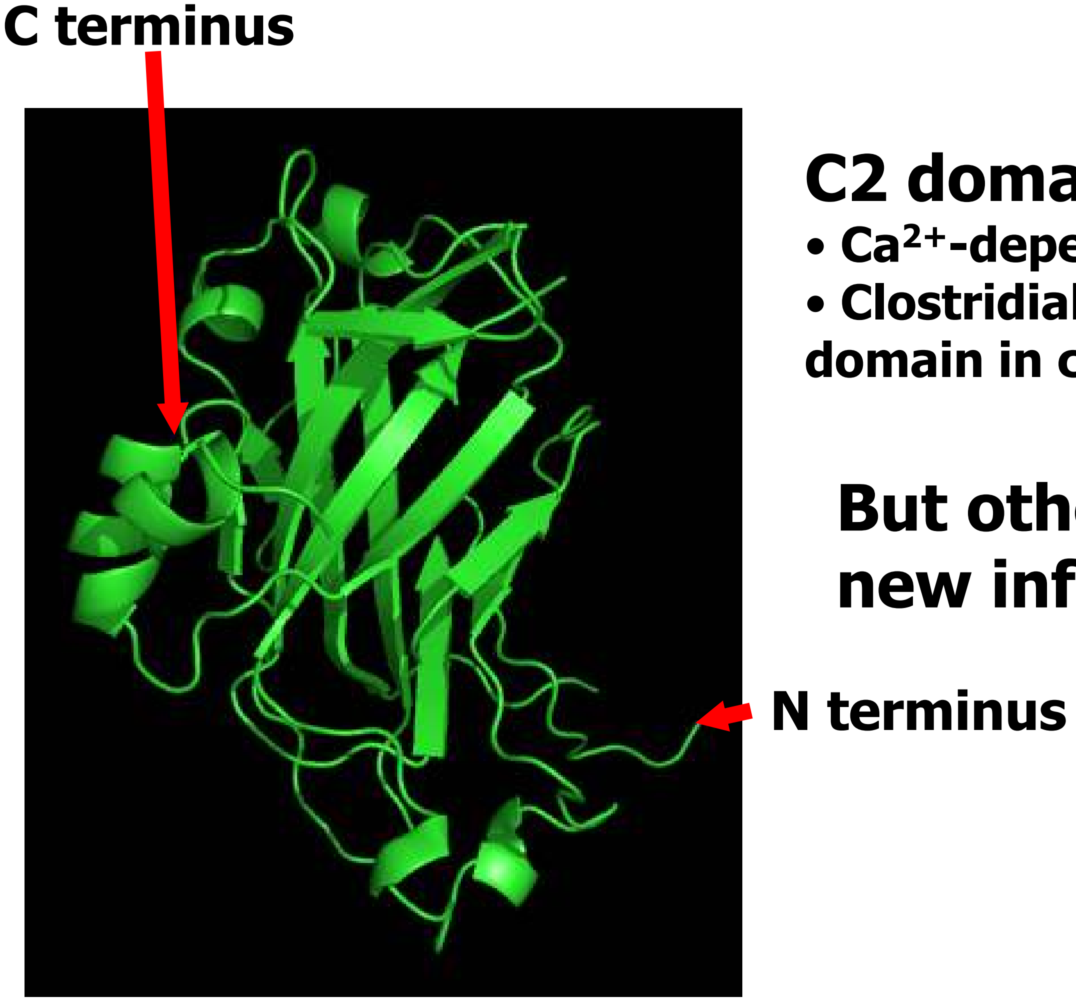


The 3 most abundant proteins: LipL32, LipL21, LipL41

# LipL32 is the most abundant protein on the leptospiral surface

- No sequence similarity to any known protein
- Will its structure give clues about function?

# Structure of LipL32



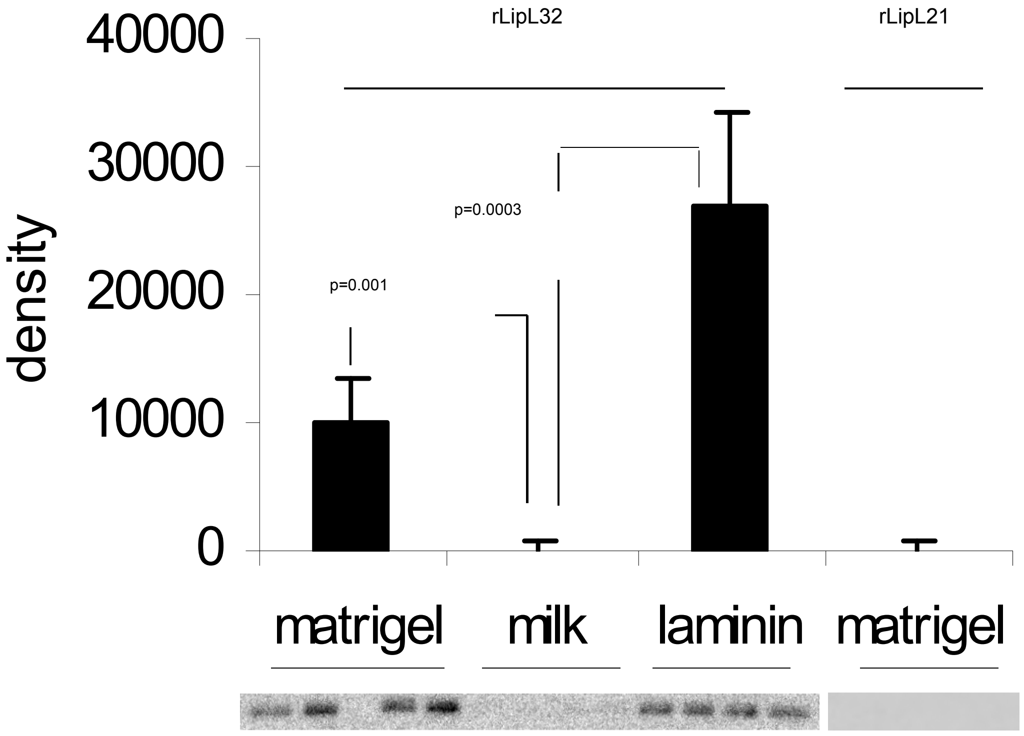
## C2 domain

- $Ca^{2+}$-dependent membrane targetting
- Clostridial α-toxins – bring catalytic domain in contact with cell membrane

**But otherwise, no real new information**

# LipL32 is the most abundant protein on the leptospiral surface

## LipL32 binds laminin



# LipL32 is a critical virulence factor?

- The most abundant OM protein
- Unique to pathogenic species
- Expressed during infection
- Binds ECM components

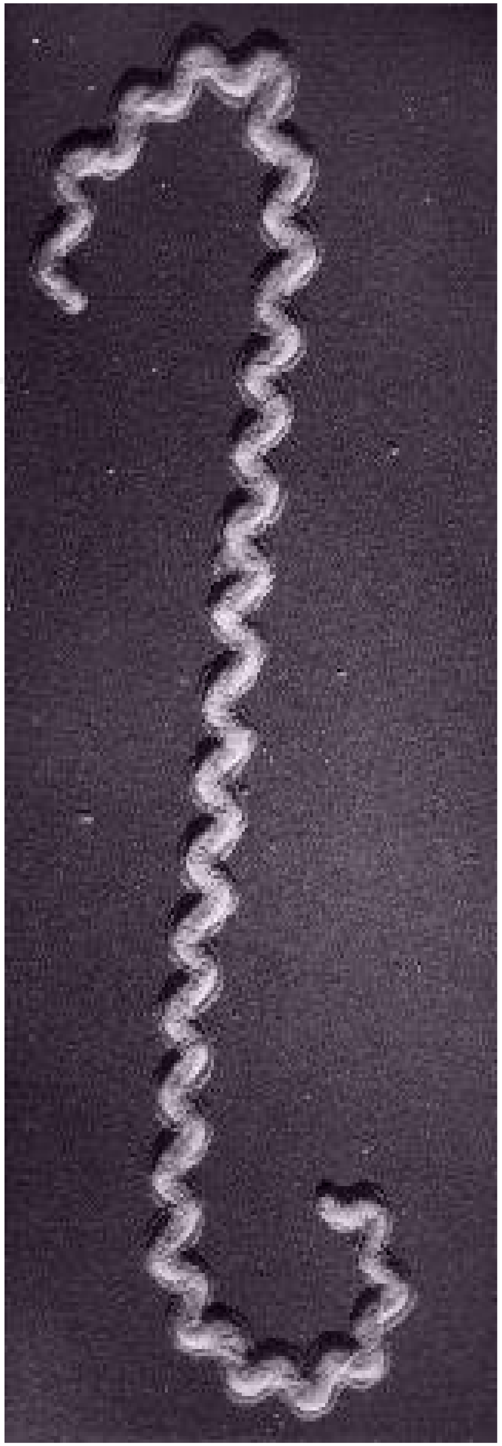
So, it must be involved in virulence, right?
Let's not jump to conclusions.

More on this later

# All info suggested pathogenic mechanisms specific to *Leptospira*

But, what are they?

The classical approach of making defined mutants was not available for *Leptospira*



# And, so, eventually…….

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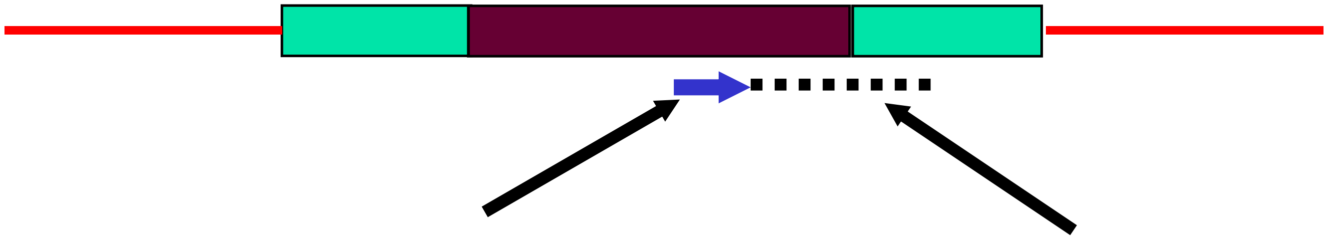
**Transposon mutagenesis system established**

# The post-genomic era: Construction of transposon mutants

genome

Random insertion of Tn

→ gene inactivation



Tn

Tn-specific primer

Direct genomic sequence

Identification of inactivated gene

# The post-genomic era:
# Construction of transposon mutants

- >1000 mutants constructed to date
- The disrupted gene identified
- ~20% intergenic
- 40% in genes of unknown function
- Phenotypic screening
- Virulence screening in hamsters

BTW, in saprophyte *L. biflexa* have many thousands of mutants
None with non-helical shape

**What might this mean?**

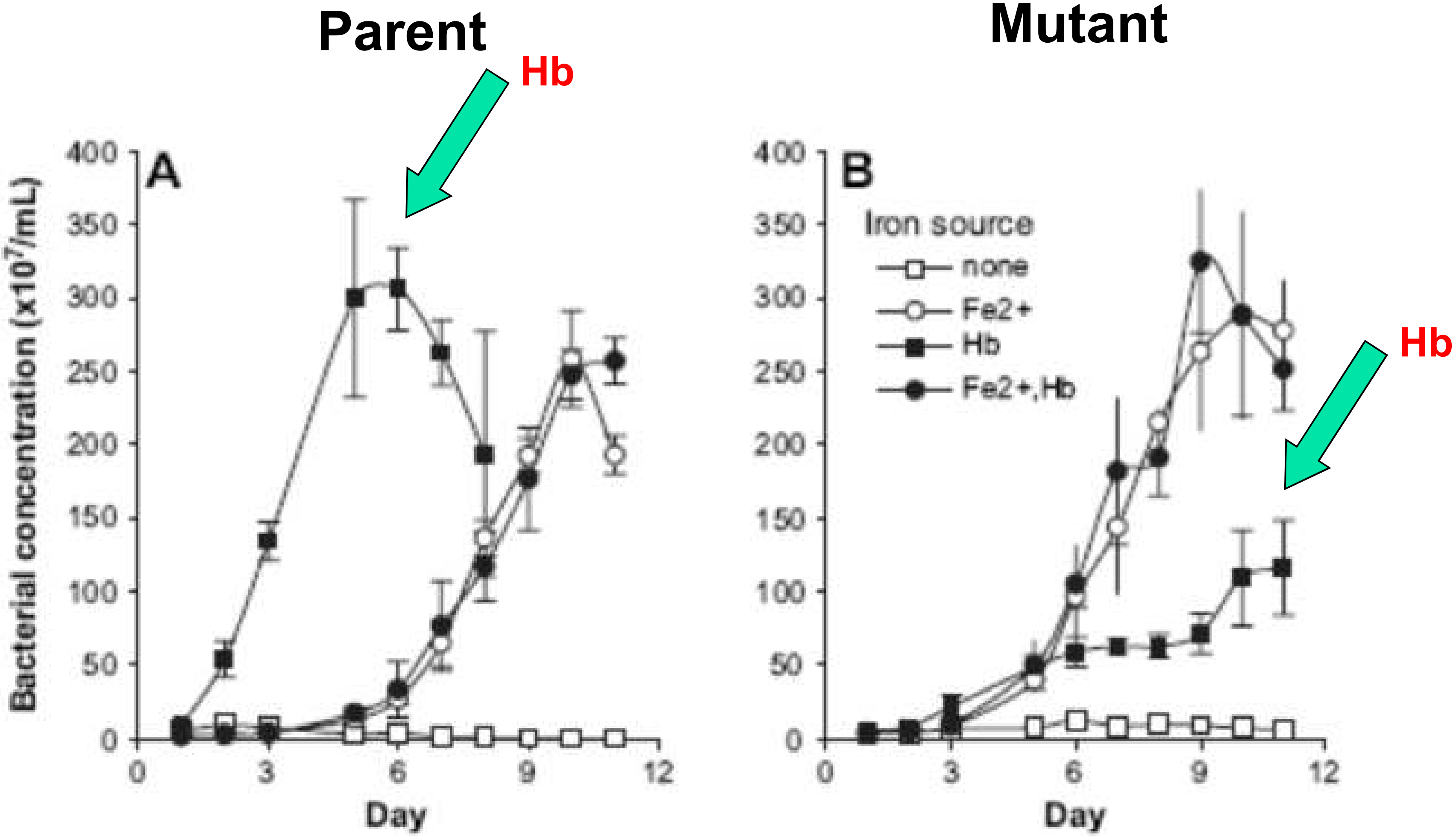
# The first surprise

## Many predicted/suspected genes not required for virulence

**OmpA-like protein**
**LA1857 Fur homologue**
**CheX**
**TonB-dependent receptor**
**Metalloprotease**
**LipL45 paralogue**
**LenB**
**LenE**
**CheB**
**LigC**

# The first functionally defined virulence gene

**Heme oxygenase mutant**



# Is the heme oxygenase mutant virulent?

| Strain | Survival | Culture | Pathology |
|---|---|---|---|
| Parent | 0/12* | (4/4) | Severe |
| Mutant | 20/24* | 23/24 | Minimal |

*$p<10^{-6}$

**Mutant survives but cannot cause disease**

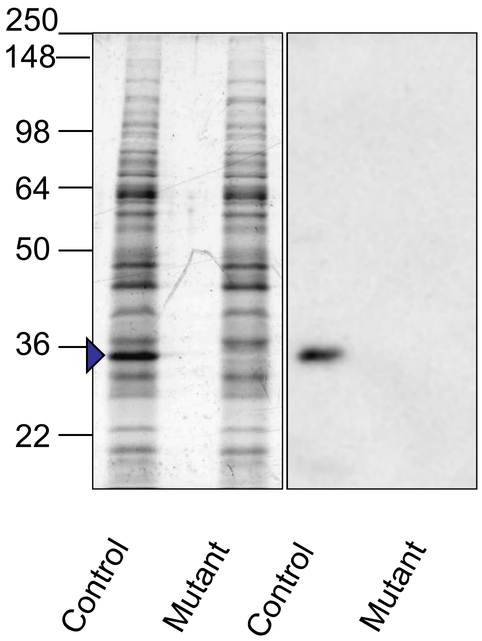
# A stroke of luck: a LipL32 mutant!!

- **The most abundant OM protein**
- **Unique to pathogenic species**
- **Expressed during infection**
- **Binds ECM components**

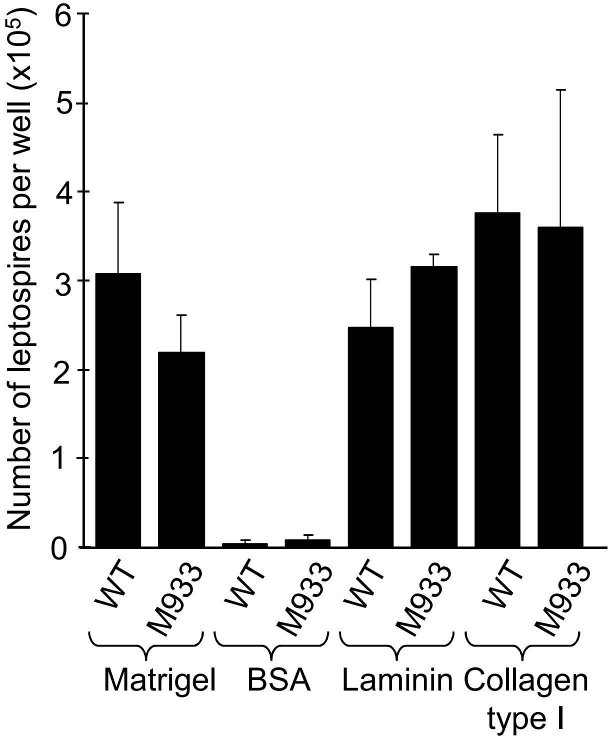
**So, it must be involved in virulence, right?**
**Let's not jump to conclusions.**

**More on this later (later is now)**

# A stroke of luck: a LipL32 mutant!!

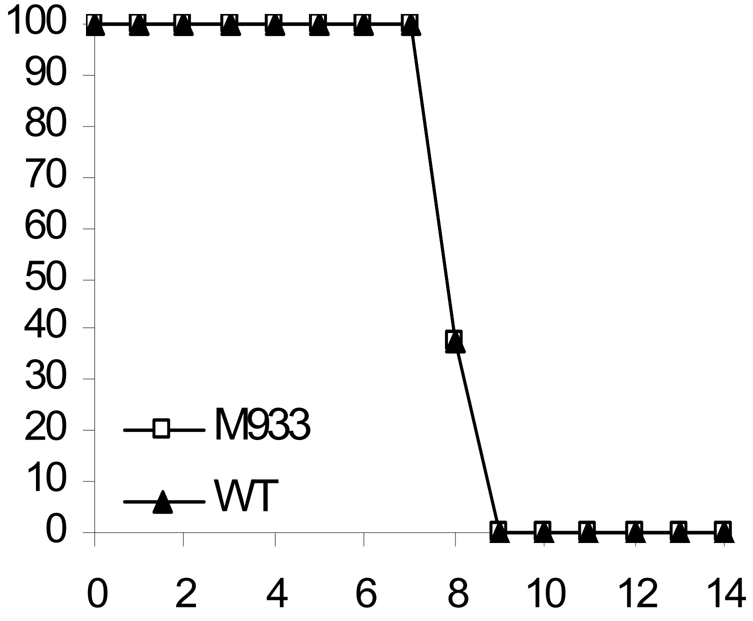


**Confirm phenotype**

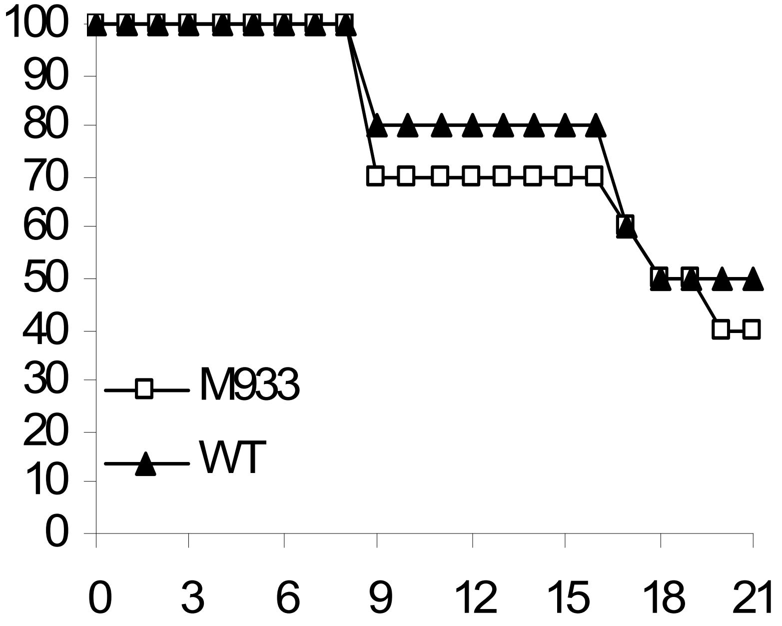


**Mutant binds ECM**

# Virulence of LipL32 mutant
# Acute infection

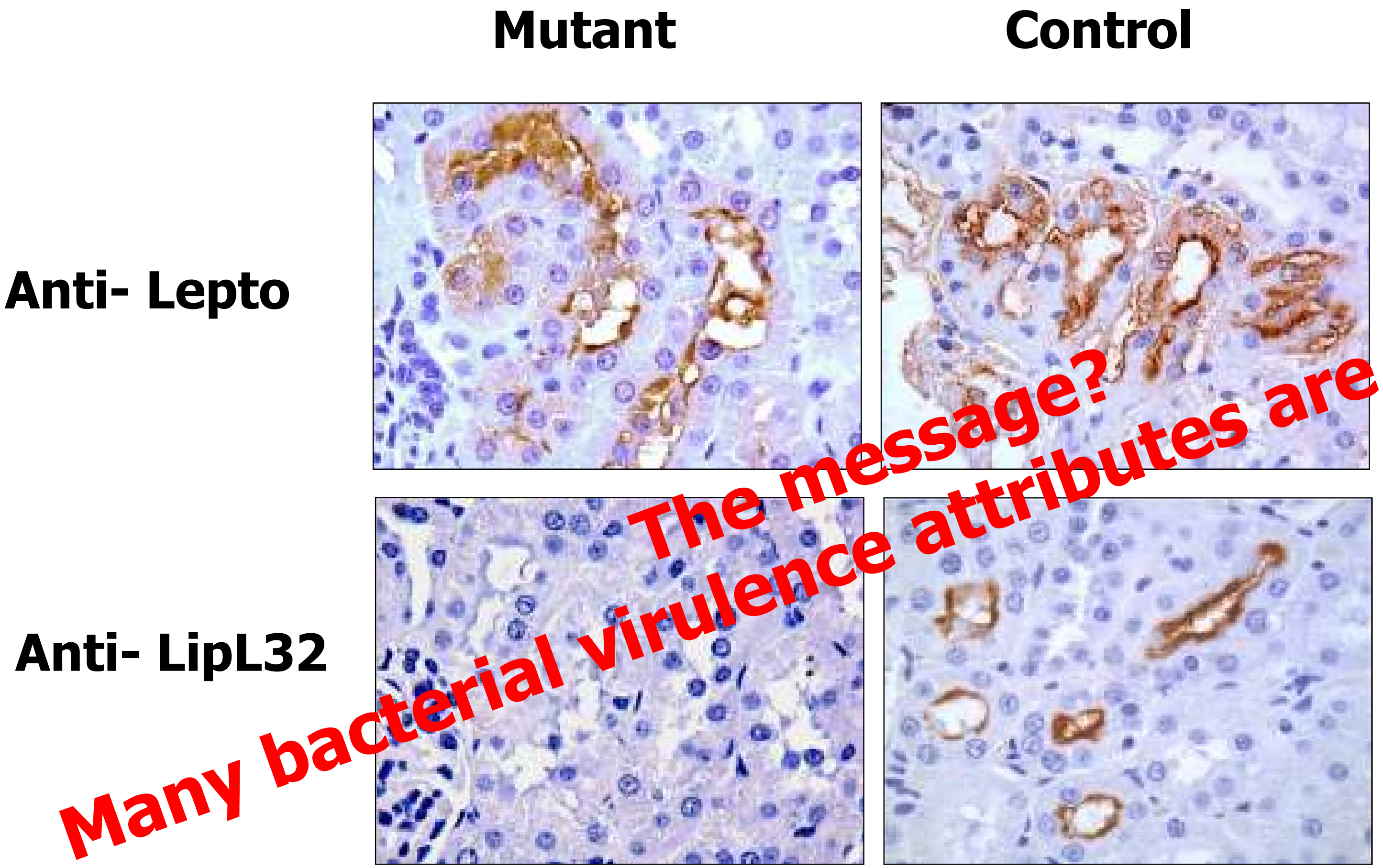


Systemic infection



Ocular infection

# Virulence of LipL32 mutant Kidney colonisation



The message?

Many bacterial virulence attributes are redundant

Kidney colonisation

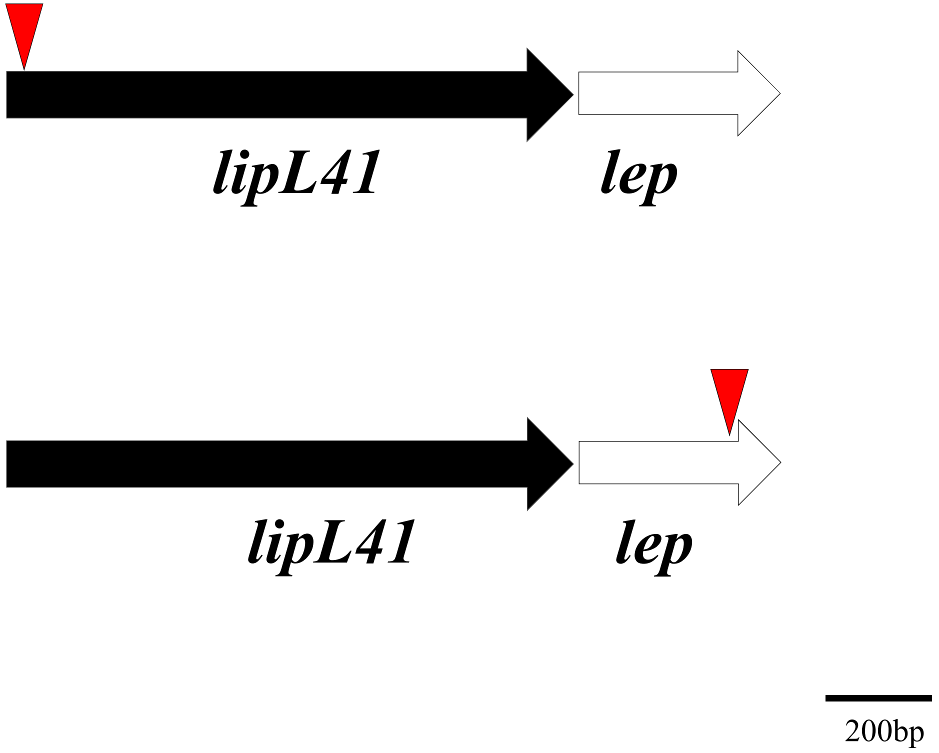
# Question

**What experiments could be conducted to define a function for LipL32?**

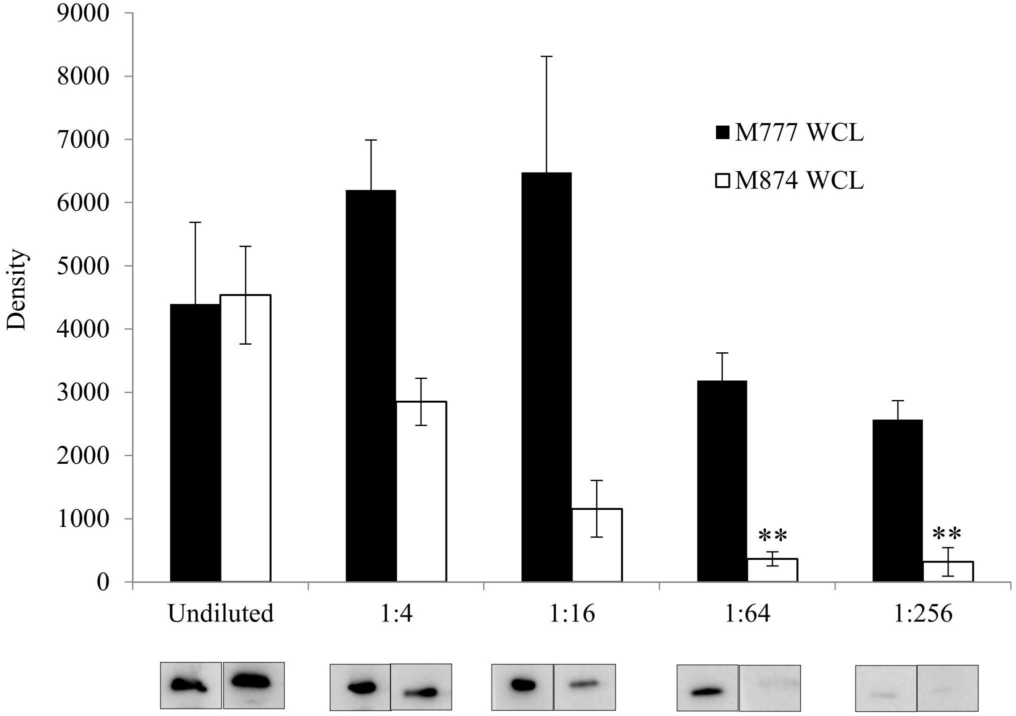
# Question

**What about LipL41?**
**It’s the 3rd most abundant surface protein**

# Two handy mutants

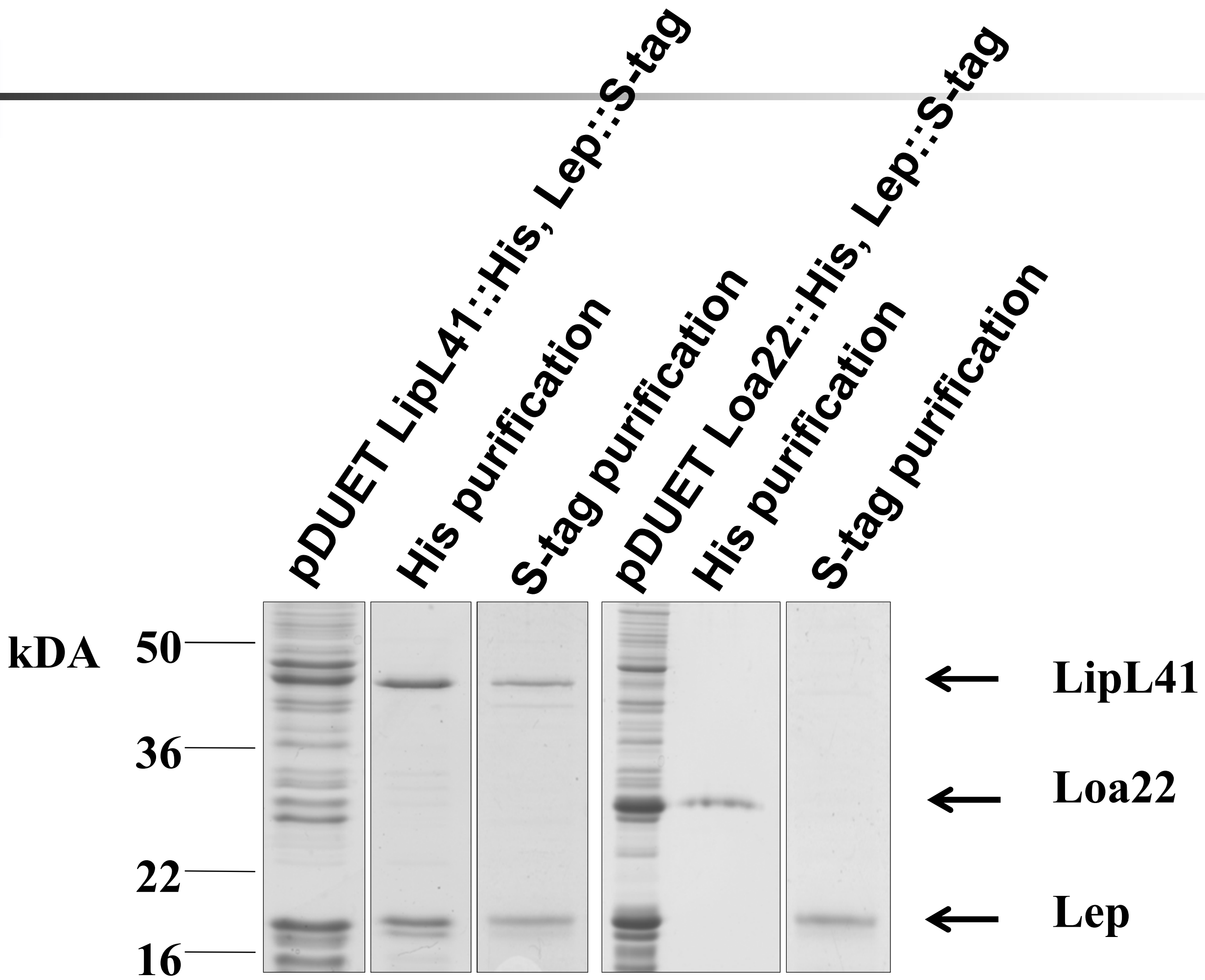


# Surprise!
# Lep mutant has >10-fold reduction in LipL41

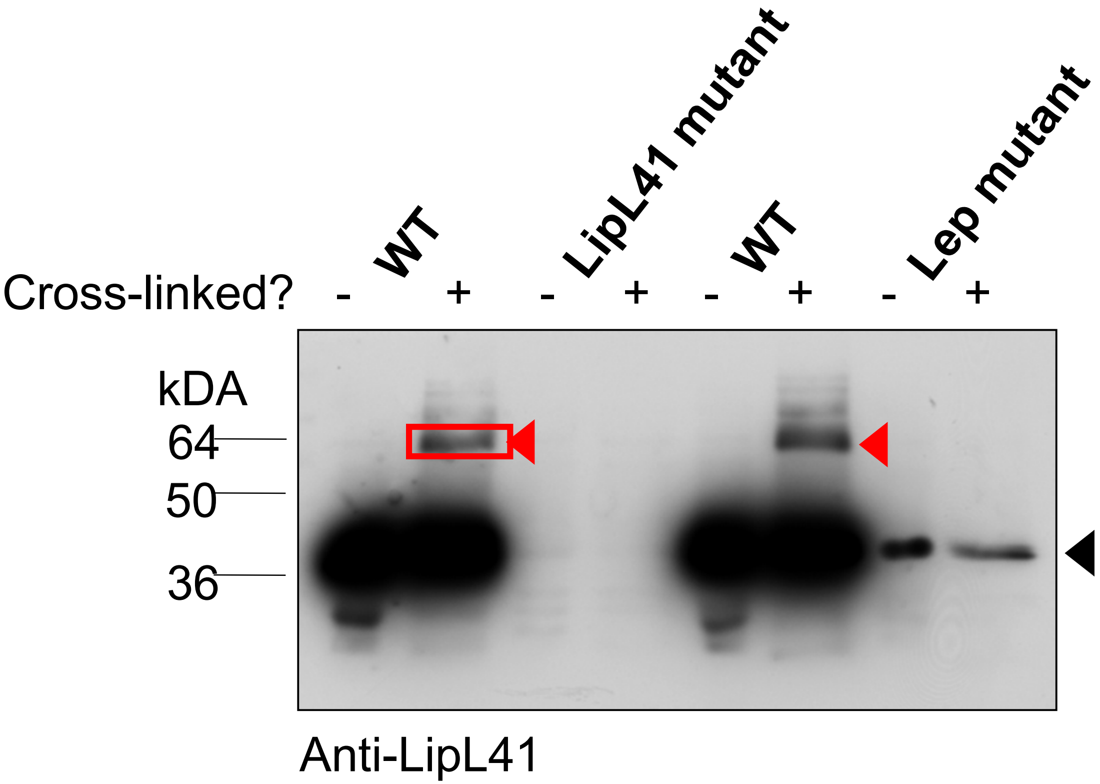


**(It's not transcriptional)**

# Lep and LipL41 co-purify



# Cross linking with DTBP



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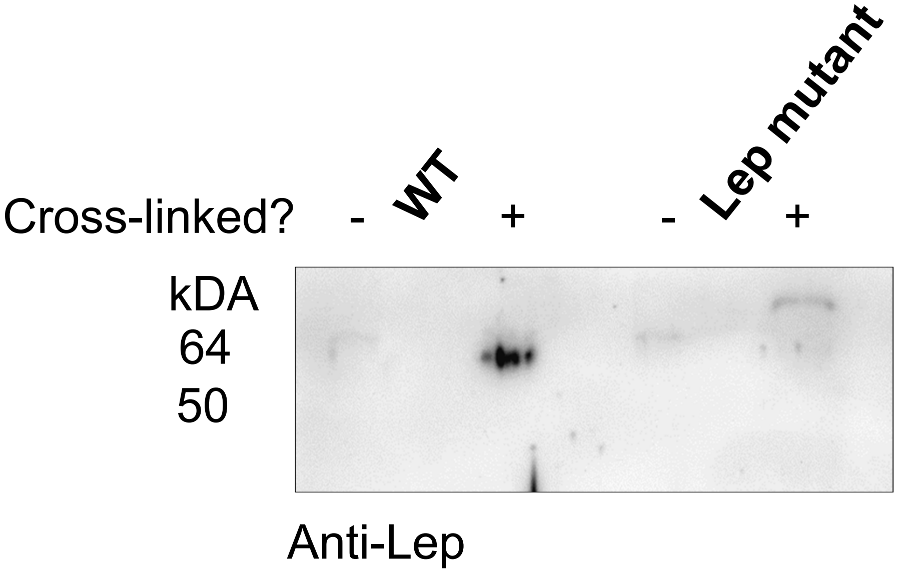


# Are the mutants attenuated?

So LipL41 remains an enigma

- very abundant
- only in pathogens
- function unknown?
- not required for virulence

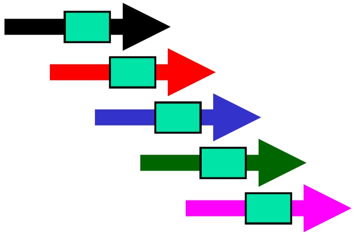
**Many bacterial virulence attributes are redundant**

# The post-genomic era:
# Screening of transposon mutants

- Direct hamster challenge with individual mutants

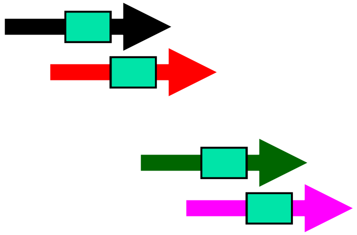
# The post-genomic era:
# Screening of transposon mutants

- Challenge with pools of mutants



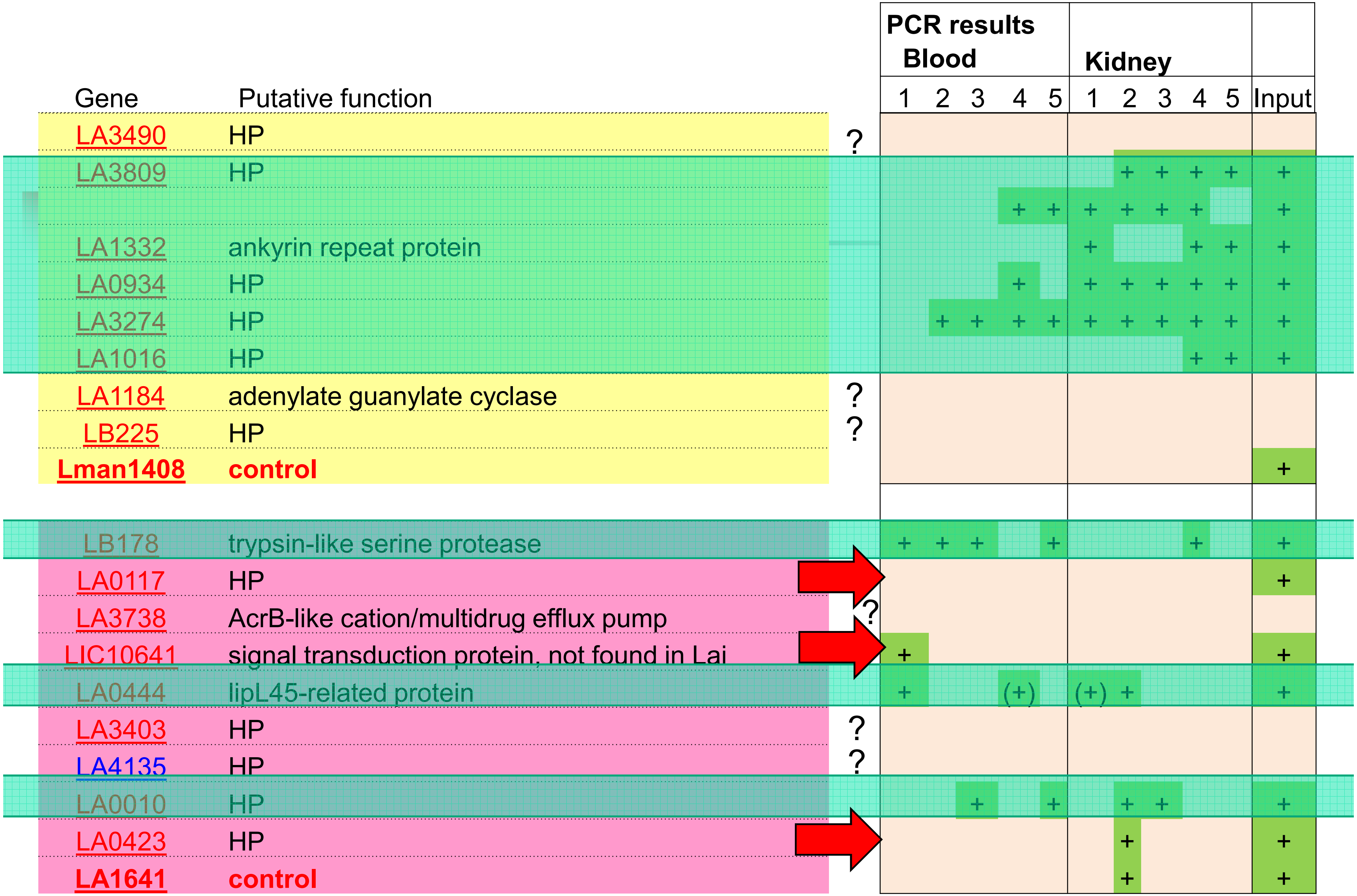
Infect with pool
of mutants

(input pool)



Collect blood
PCR for each disrupted gene

(output pool)



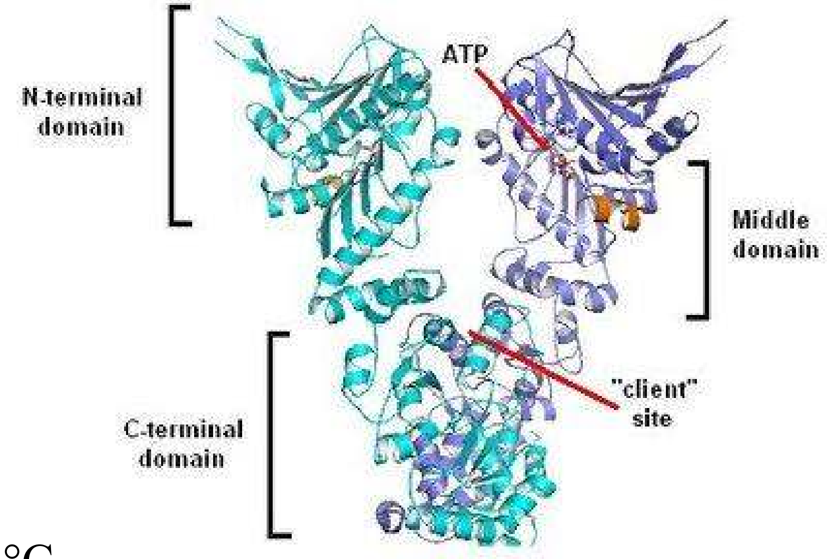
| | | | PCR results<br>Blood | | | | | Kidney | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Gene | Putative function | | 1 | 2 | 3 | 4 | 5 | 1 | 2 | 3 | 4 | 5 | Input |
| LA3490 | HP | ? | | | | | | | | | | | |
| LA3809 | HP | | | | | | | | + | + | + | + | + |
| | | | | | | + | + | + | + | + | + | | + |
| LA1332 | ankyrin repeat protein | | | | | | | + | | | + | + | + |
| LA0934 | HP | | | | | + | | + | + | + | + | + | + |
| LA3274 | HP | | | + | + | + | + | + | + | + | + | + | + |
| LA1016 | HP | | | | | | | | | | + | + | + |
| LA1184 | adenylate guanylate cyclase | ? | | | | | | | | | | | |
| LB225 | HP | ? | | | | | | | | | | | |
| **Lman1408** | **control** | | | | | | | | | | | | + |
| | | | | | | | | | | | | | |
| LB178 | trypsin-like serine protease | | + | + | + | | + | | | | + | | + |
| LA0117 | HP | | | | | | | | | | | | + |
| LA3738 | AcrB-like cation/multidrug efflux pump | ? | | | | | | | | | | | |
| LIC10641 | signal transduction protein, not found in Lai | | + | | | | | | | | | | + |
| LA0444 | lipL45-related protein | | + | | | (+) | | (+) | + | | | | + |
| LA3403 | HP | ? | | | | | | | | | | | |
| LA4135 | HP | ? | | | | | | | | | | | |
| LA0010 | HP | | | | + | | + | | + | + | | | + |
| LA0423 | HP | | | | | | | | + | | | | + |
| **LA1641** | **control** | | | | | | | | + | | | | + |

# It’s not all negative

## High temperature protein G (HtpG)

- HtpG
    - Bacterial homolog to Hsp90
    - Acts as a dimer
    - 3 domains: N-, M-, and C-domain

- Molecular chaperone associated with heat shock
    - Essential protein in eukaryotes
    - Different phenotypes in bacterial species
        - *E. coli* – growth defect at 44°C
        - *B. subtilis* – growth unaffected up to 48°C



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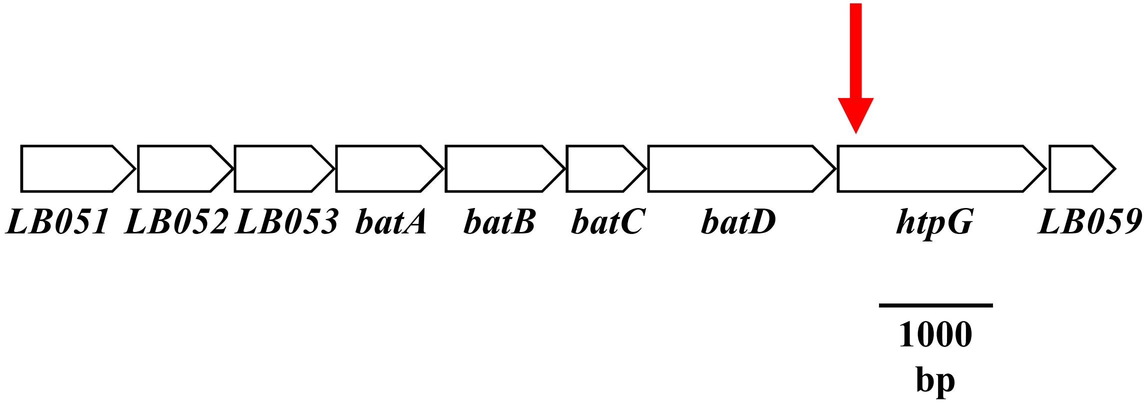
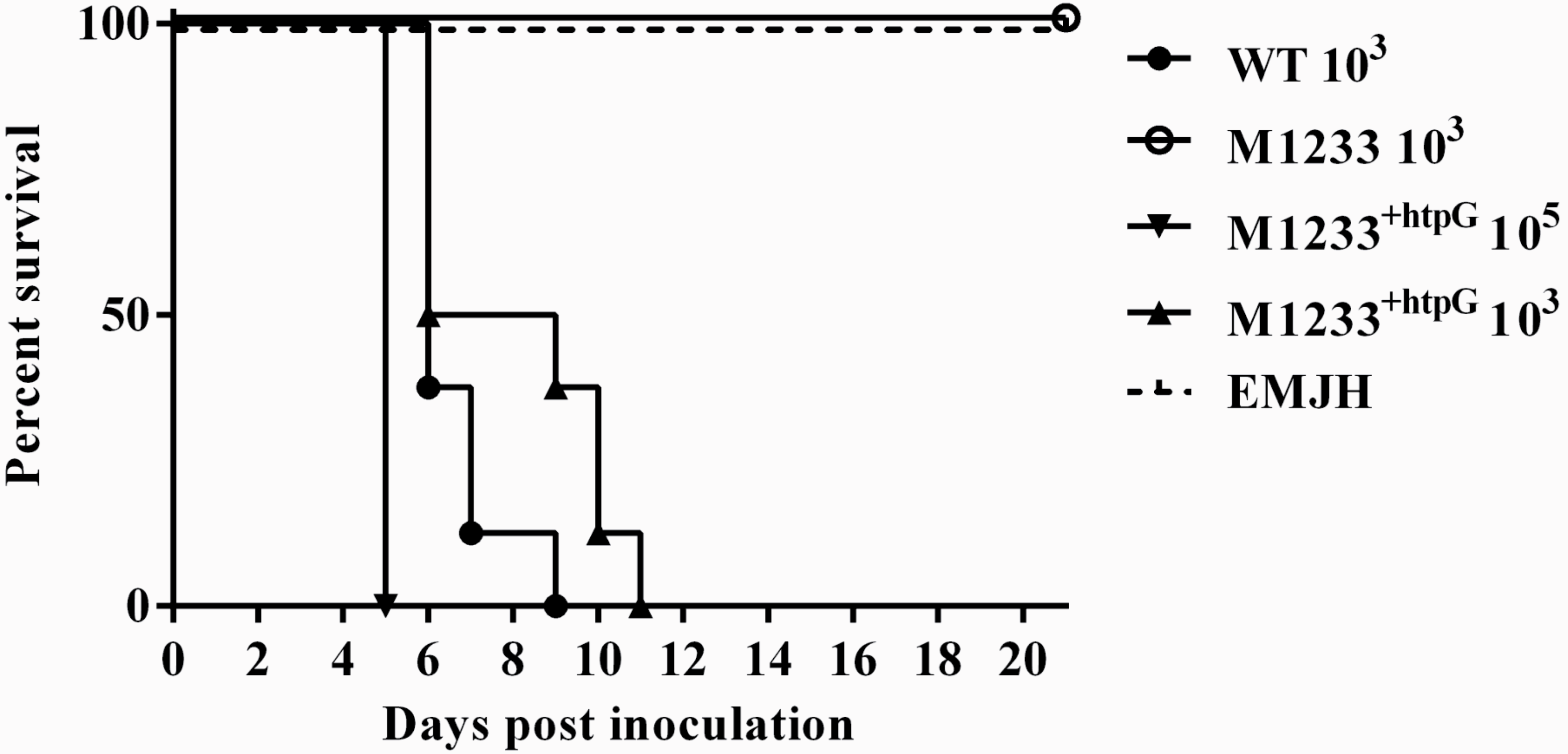


The putative *htpG* (Hsp90) operon

# Virulence of *htpG* mutant



Mutant colonises hamster kidneys

# Some interesting things about *htpG*

- **Mutant colonises hamster kidneys**
- **Different effects in different species**
    - *E. coli* – growth defect at 44°C
    - *B. subtilis* – growth unaffected up to 48°C
    - Attenuated in *F. tularensis* and *E. tarda*
    - Not attenuated in *P. gingivalis*
- ***Leptospira htpG*** **mutant shows no difference in:**
    - *In vitro* growth, motility, LPS
    - Sensitivity to heat, osmotic, pH stress, complement resistance
    - Survival in macrophages
- **HtpG has a paralog, LA1231 (43% similarity)**
    - *la1231* mutant retains virulence
    - Transcription studies heat shock:
        - *htpG* – no change
        - *la1231* – 2.6-fold increase

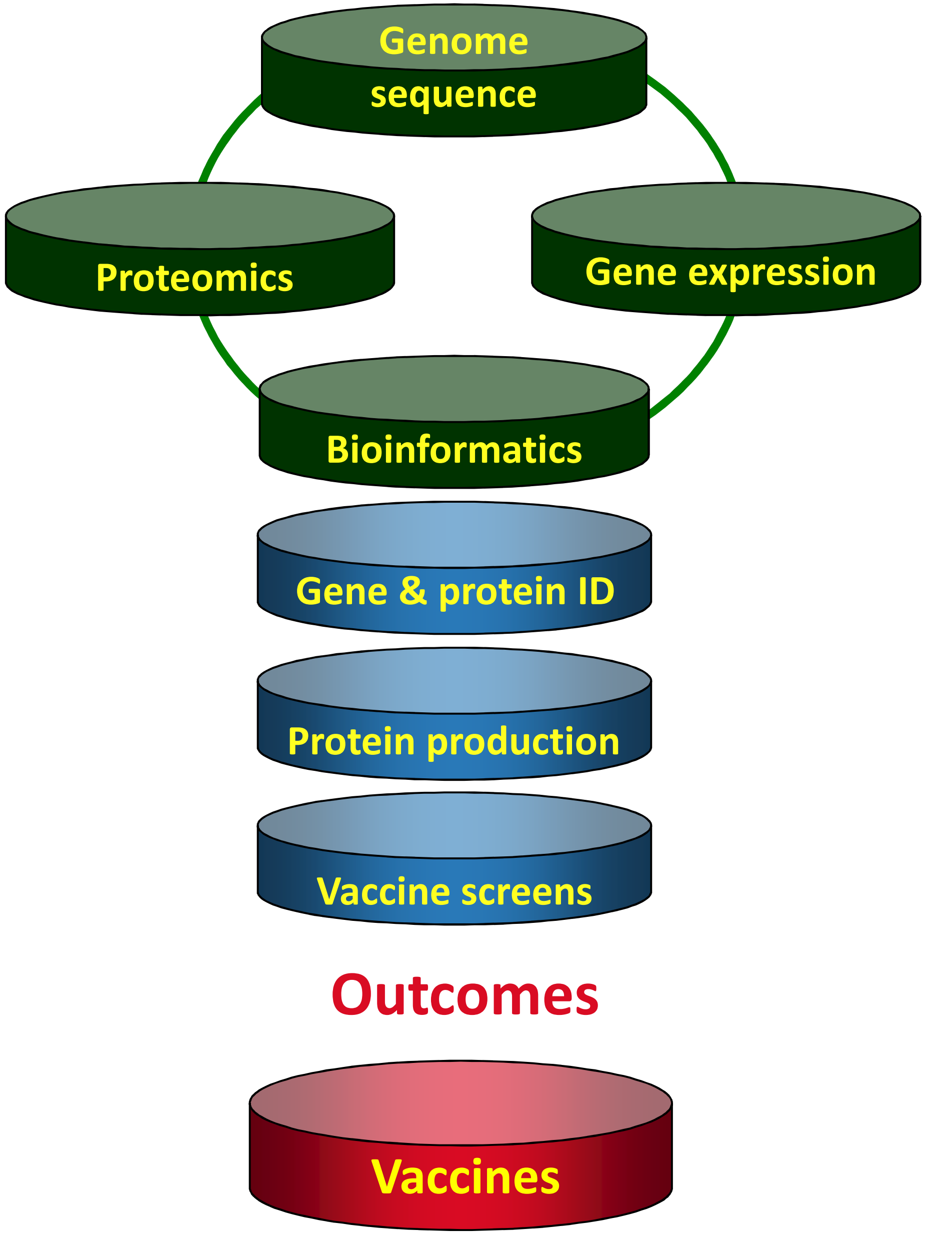
# Nevertheless:
# some essential virulence factors identified

| **Gene/feature** | **Mechanism** |
|---|---|
| LPS | Unknown |
| Flagella | Motility, but precise mechanism not known |
| *hemO* | Obtain iron from heme |
| *katE* | Catalase activity |
| *loa22* | Unknown |
| *clpB* | Stress response |
| *htpG* | Probably stress response |
| *lruA* | Interaction with Apolipoprotein A1 |
| *mce* | Cell entry? |
| *colA* | Tissue damage? |
| *lruA* | Binds apolipoprotein A1 |

# The post-genomic era:
# Vaccine development

- Killed whole cell vaccines
- Problems
  - Reactogenicity
    - Foreign proteins from growth medium
    - Inherent reactogenicity of leptospires (LPS? Lipids?)
  - Problem of using any bovine material
  - Immunity is serovar (serogroup?) specific
  - DOI is short
- Need to be tailored for:
  - Animal species
  - Geographical region

# The reverse vaccinology pipeline



Genome sequence

Proteomics

Gene expression

Bioinformatics

Gene & protein ID

Protein production

Vaccine screens

Outcomes

Vaccines

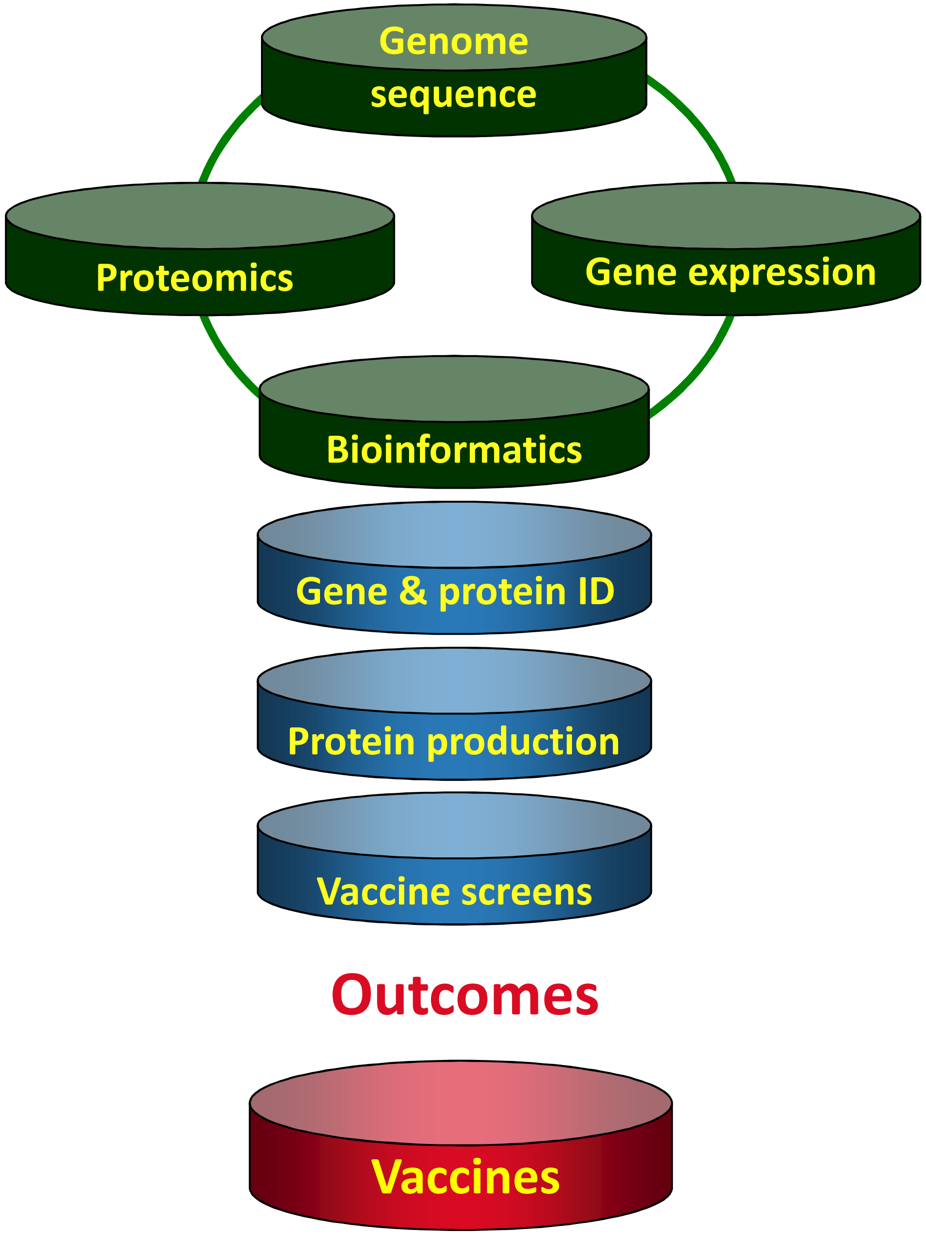
| | |
|---|---|
| **Number of Genes** | **2844**<br>Genome sequenced |
| **Suitable Genes** | **237**<br>Putative surface proteins |
| **Expressed Proteins** | **~200**<br>Vaccine candidates purified |

# Protein production: the first problem

- 10% soluble
    - Purified as soluble proteins

- 90% of proteins insoluble
    - Purified as denatured proteins

# The reverse vaccinology pipeline



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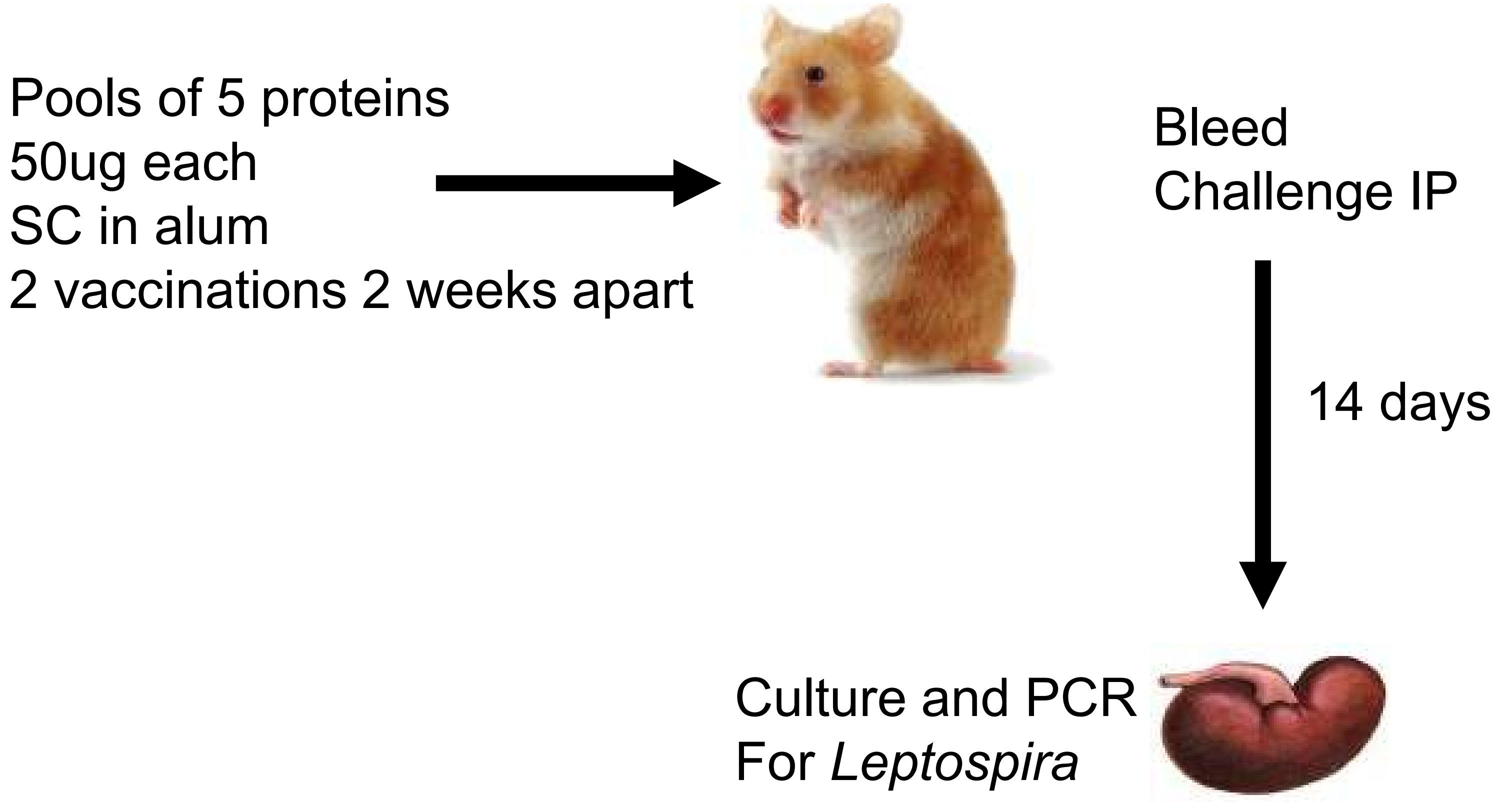
# The hamster infection model
# Serovar Hardjo – non lethal



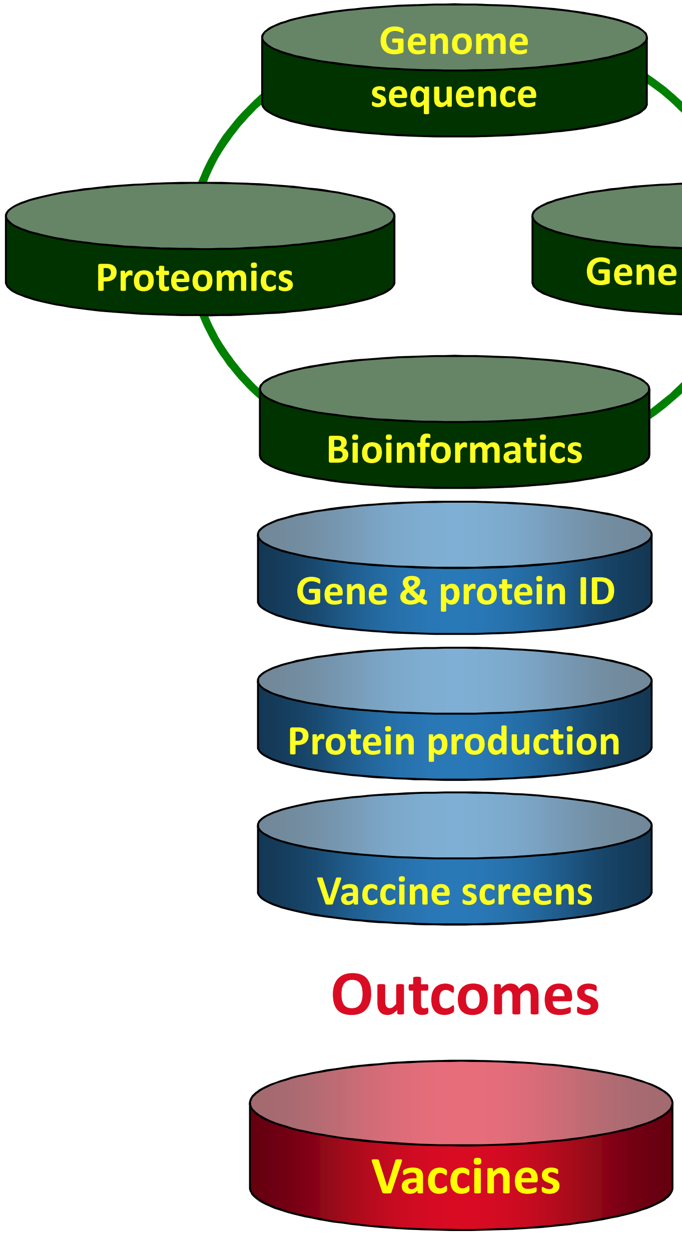
Pools of 5 proteins
50ug each
SC in alum
2 vaccinations 2 weeks apart

Bleed
Challenge IP

14 days

Culture and PCR
For *Leptospira*

# The reverse vaccinology pipeline



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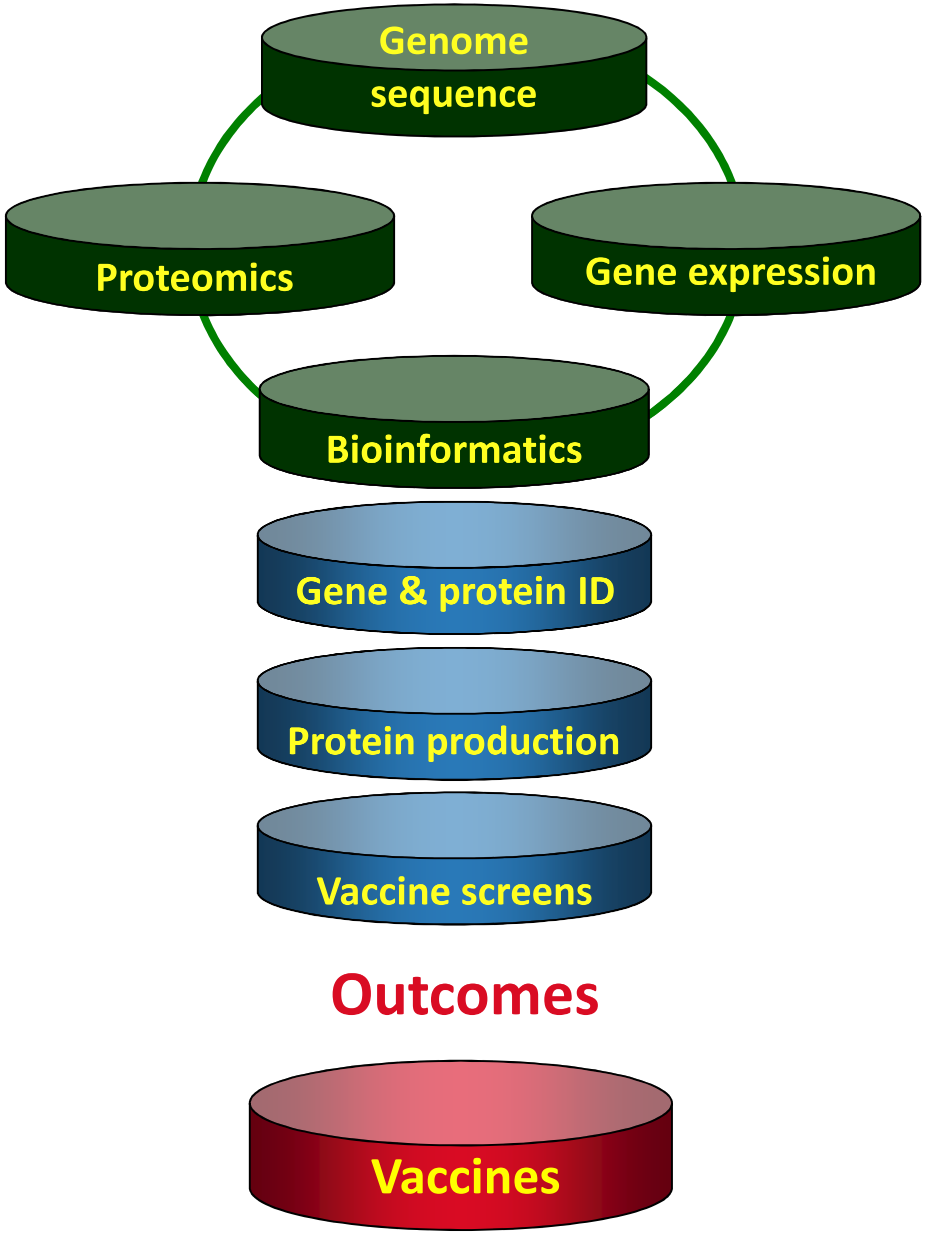
| **Proven Antigens** | **>95%**<br>Immune response |

# Antibody response: the second problem

- **>95% of sera reacted with corresponding recombinant protein**
- **~50% reacted with the protein in *Leptospira* (possible reasons?)**
- **Test all antigens for protection**

# The reverse vaccinology pipeline



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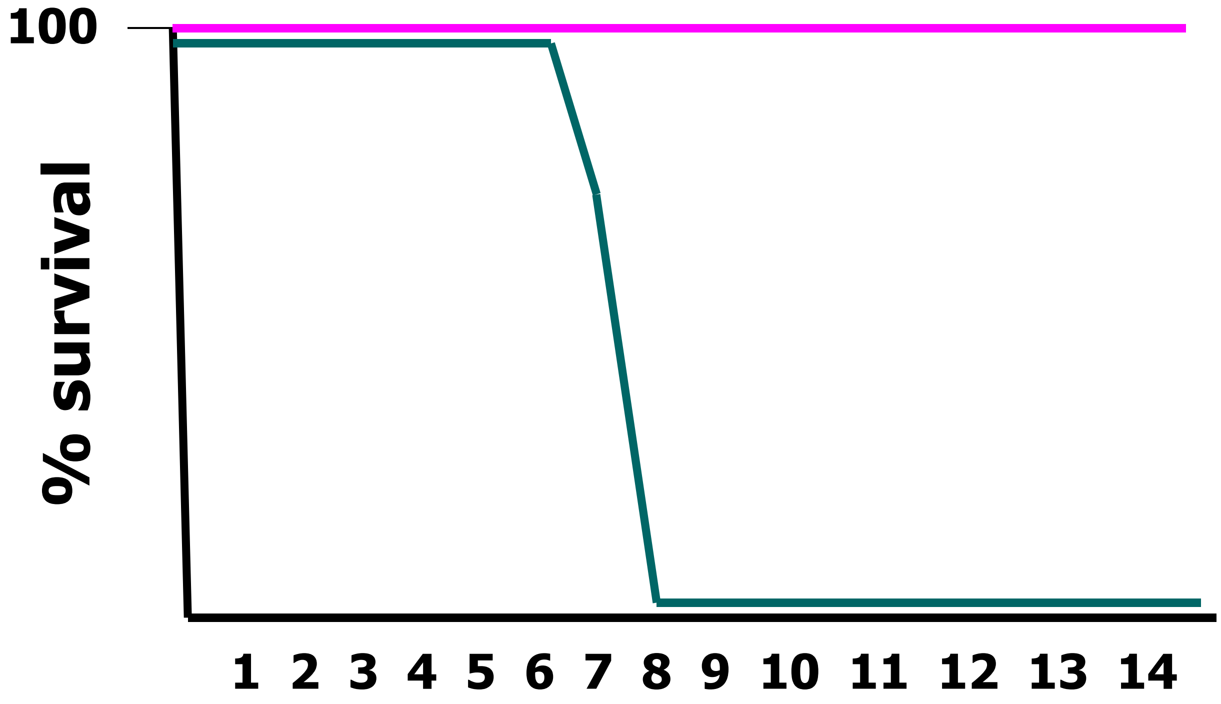
| **Vaccine Trials** | **~200**<br>Vaccine candidates tested |
| **Proven Antigens** | **>95%**<br>Immune response |
| **Lead Candidates** | **00000000**<br>Potentially protective antigens |

# Mutant screen: a vaccine spin off

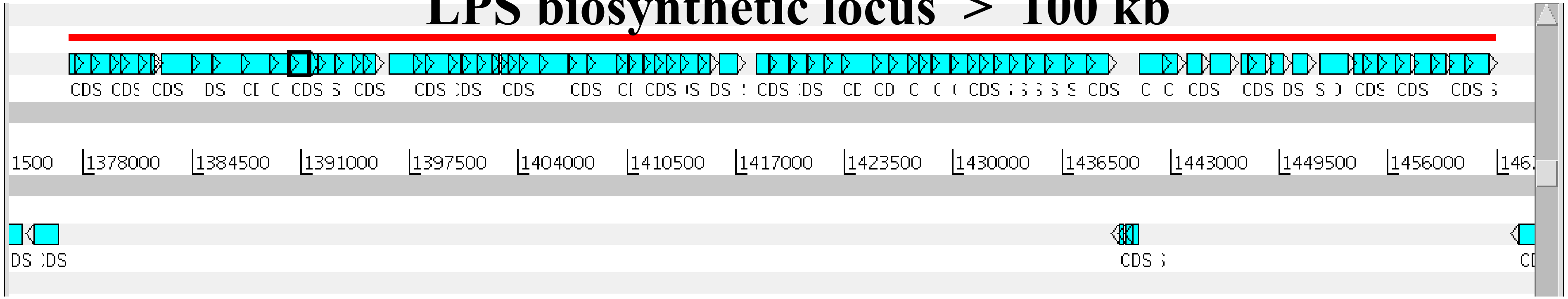
## An attenuated mutant

Hamster survival

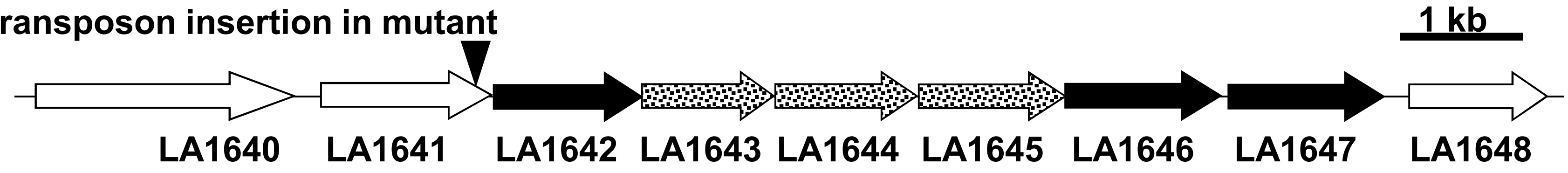


# Attenuated mutant: altered LPS

LPS biosynthetic locus > 100 kb



Transposon insertion in mutant

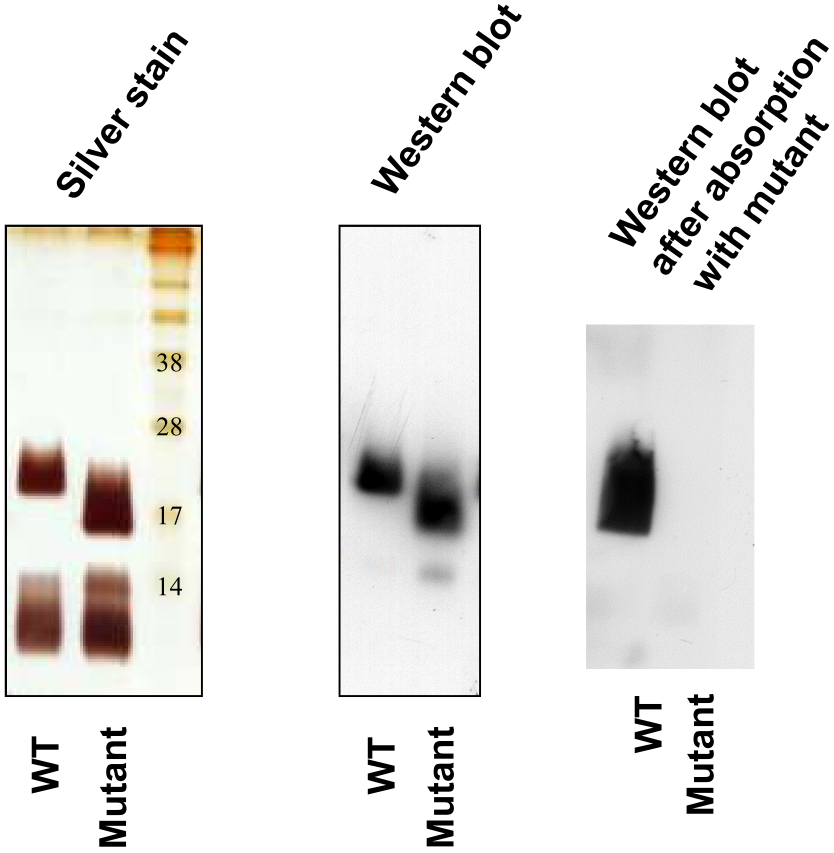


■ Glycosyltransferase

▨ Sugar epimerase/isomerase

□ No predicted function

# Attenuated mutant: altered LPS

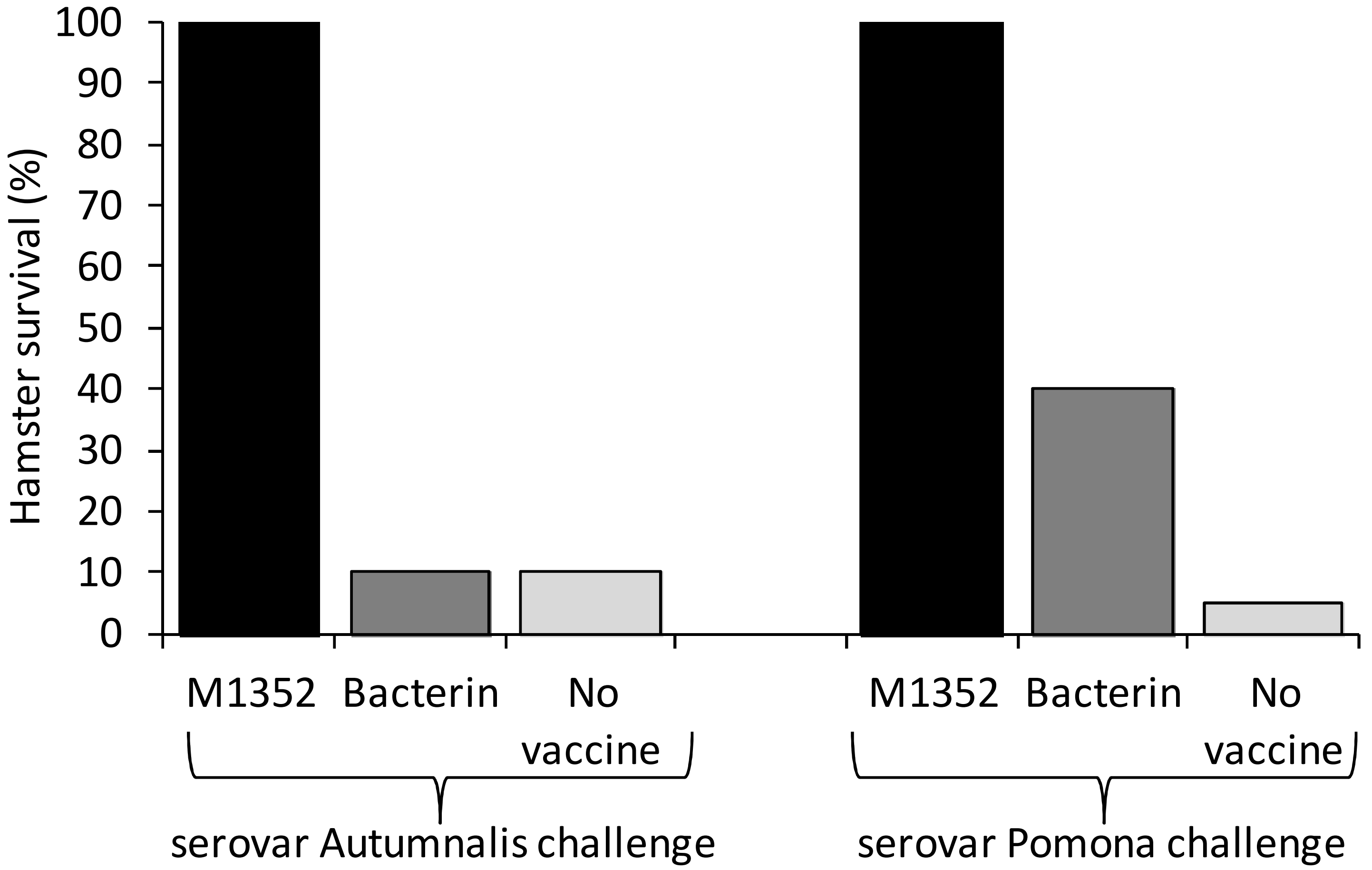


So, LPS is present but some (unknown) epitopes are missing

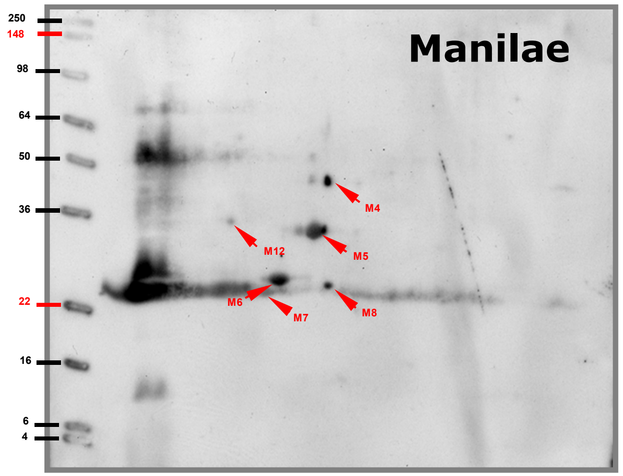
# Vaccine spin off
# LPS mutant: can it induce protection? (homologous challenge - Manilae)

| Vaccine | Hamster survival | Kidney +ve |
|---|---|---|
| Control | 0/20 | 20/20 |
| Killed bacteria | 17/20 | 11/20 |
| LPS mutant | 20/20 | 0/20 |

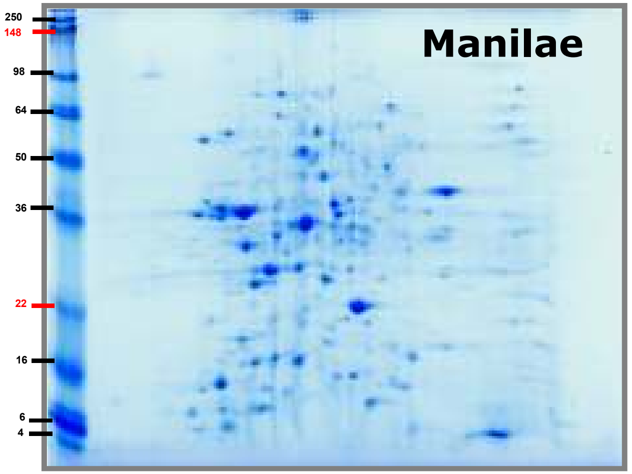
# LPS mutant: can it induce protection? (heterologous challenge)

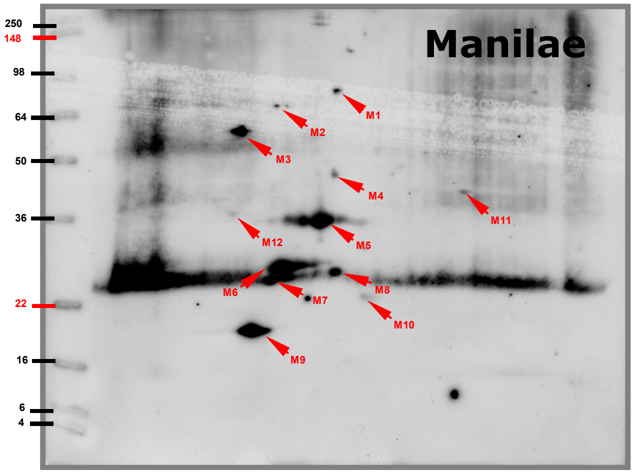


# What antibodies are elicited by the LPS mutant compared to killed leptospires?

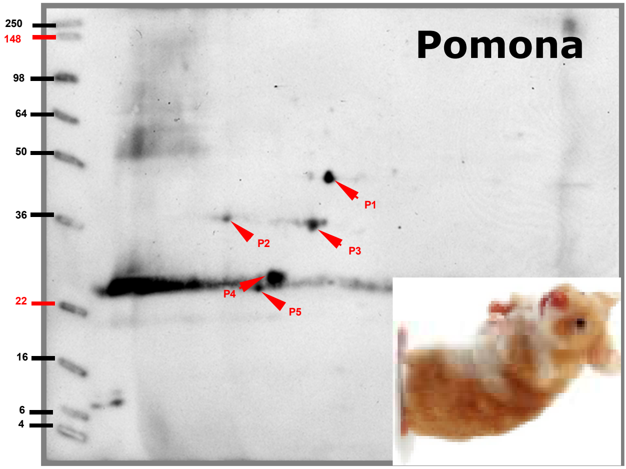


Immunised with killed leptos

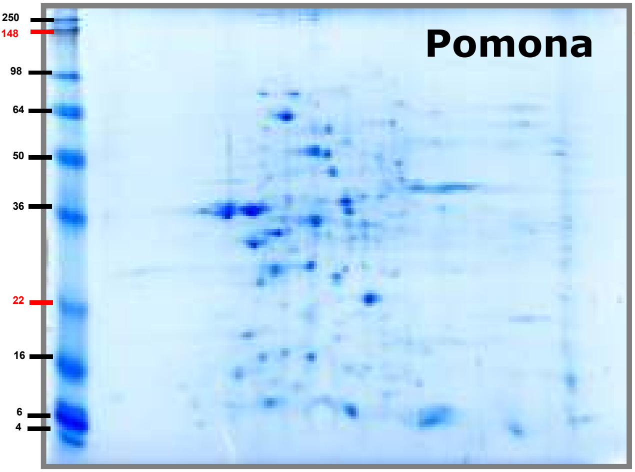


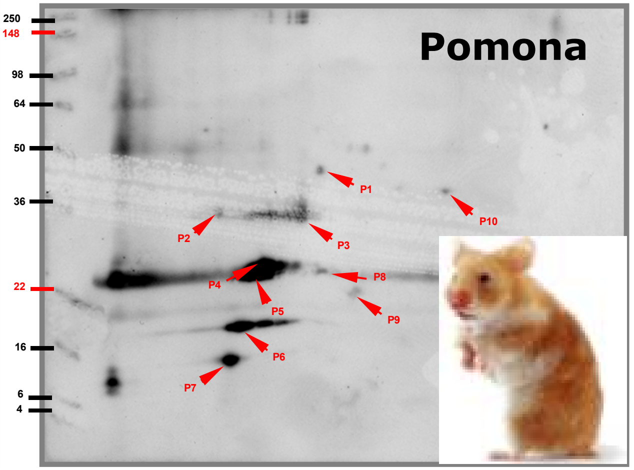


Immunised with live mutant



Immunised with killed leptos





Immunised with live mutant

# Some key questions

- **What antigens are differentially recognised by protected hamsters?**
- **What is the limitation of this approach?**
- **How could this be overcome?**

# Now to the mice: why?

- **Acute leptospirosis in humans and hamsters is an accidental, dead-end infection**

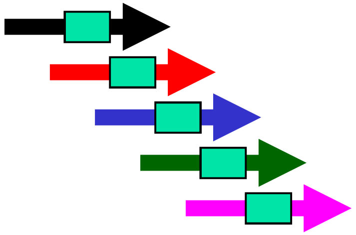
- **The natural evolutionary niche is the rodent kidney**

- **<10cfu in hamster – lethal infection**

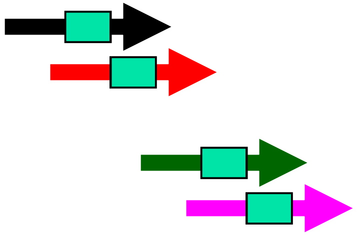
- **$10^8$ cfu in rat/mouse – asymptomatic renal carriage**

# Screening of transposon mutants

- Infect mice with pools of mutants



Infect with pool
of mutants



After 2 weeks, collect kidney
PCR for each disrupted gene
Check each mutant individually
(PCR and culture)

# Colonisation-deficient mutants

| Gene | Function | Attenuated in acute model? |
|---|---|---|
| *la1641* | LPS biosynthesis | Yes |
| *htpG* | Chaperone | Yes* |
| *la0589* | Unknown | No |
| *la0969-75* | ABC transporter | No |
| *la2786* | Unknown | No |
| *lb191* | TonB-dep receptor | No |
| *lb194* | Unknown | No |

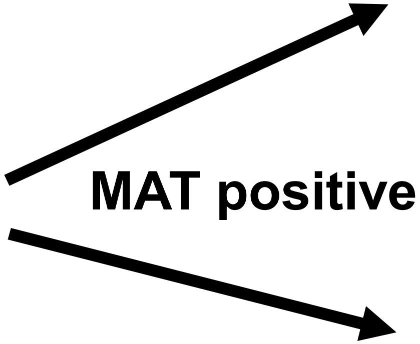
* but colonised hamster kidneys

# Conclusions

- Leptospiral virulence factors are different
  - Spirochetes diverged early in evolution
- High degree of functional redundancy
  - Single mutants may not show phenotype
- Treat results with recombinant proteins with caution
  - Biological significance may be doubtful
- Different attributes for acute infection and renal colonisation
- Bacteria (or 3 billion years of evolution) are cleverer than most microbiologists


Thank you very much!
Molto grazie!

# Mechanisms of immunity
# The curious case of the cow



Hamster protected

MAT positive

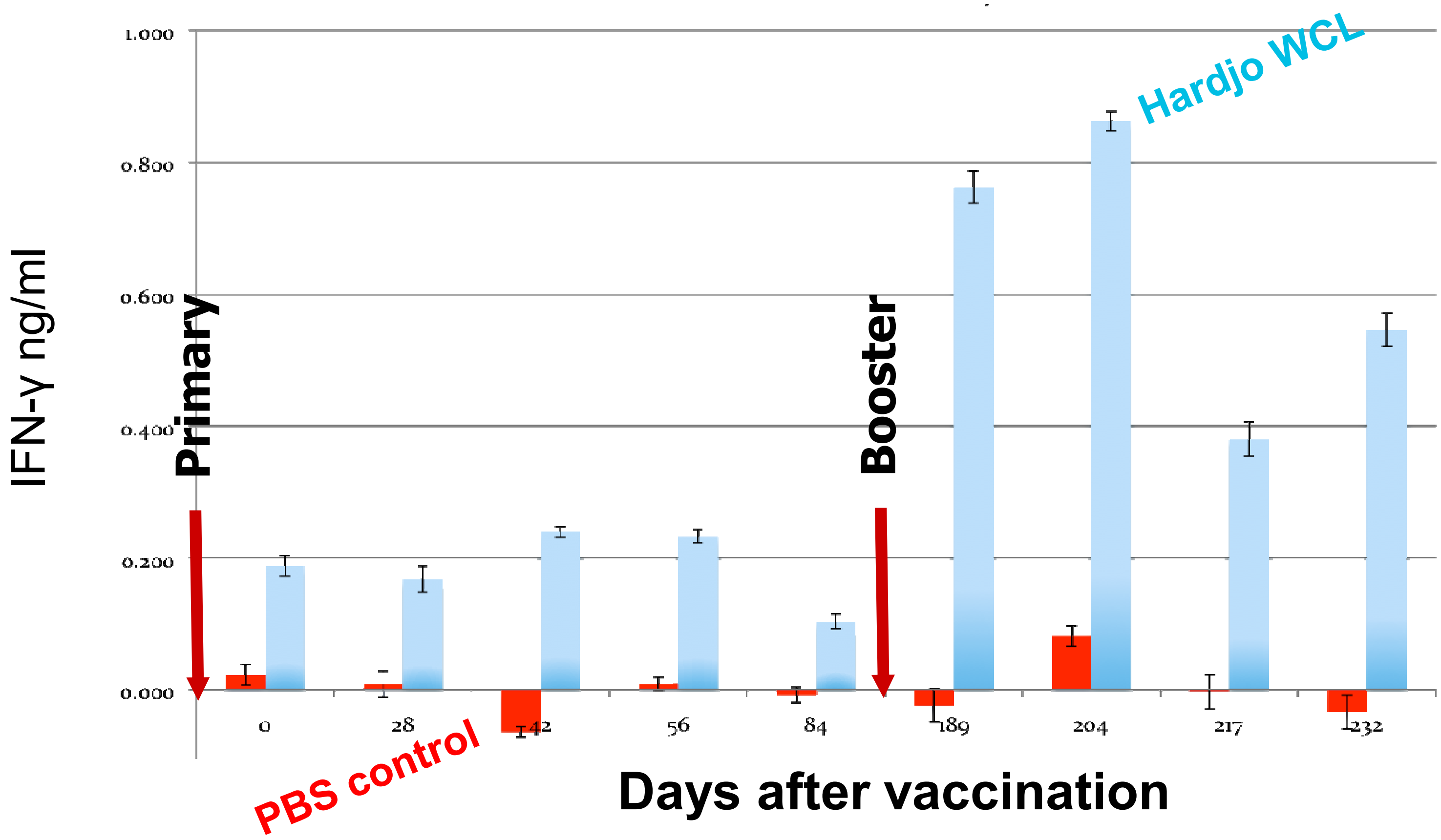
Cow NOT protected

# Mechanisms of immunity
# The curious case of the cow

**In cattle, immunity correlates with IFN-γ release**

- **Vaccinate with known IFN-γ inducing vaccine**
- **Clone, express, purify 238 Hardjo proteins**
- **Test each for ability to stimulate IFN-γ release in blood of vaccinated cows**

# Mechanisms of immunity
# The curious case of the cow



# Mechanisms of immunity
# The curious case of the cow

## Which proteins can stimulate IFN- release?