Horserace Betting Levy Board
5th Floor
21 Bloomsbury Street
London WC1B 3HF

Tel: 020 7333 0043
Fax: 020 7333 0041
Web: www.hblb.org.uk
Email: equine.grants@hblb.org.uk

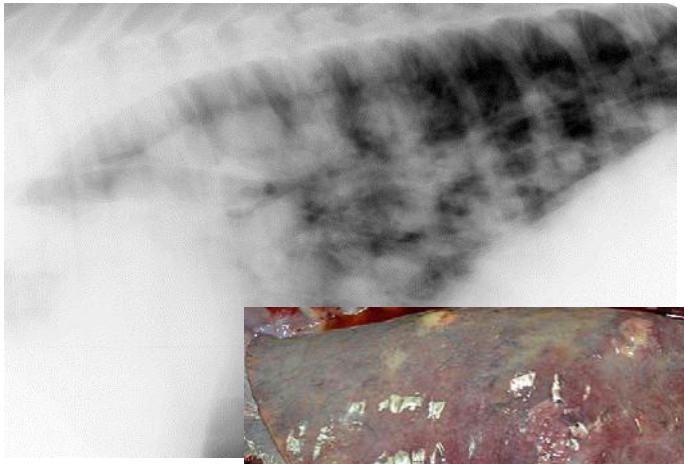

# Functional analysis of the *Rhodococcus equi* genome

The International *Rhodococcus equi* consortium (IREC)
"*Rhodococus equi*: from genome to function"
Prof Jose Vazquez-Boland
University of Edinburgh

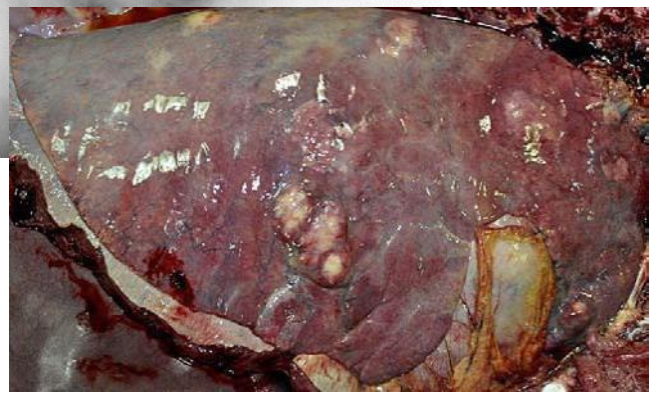
Prj: 753

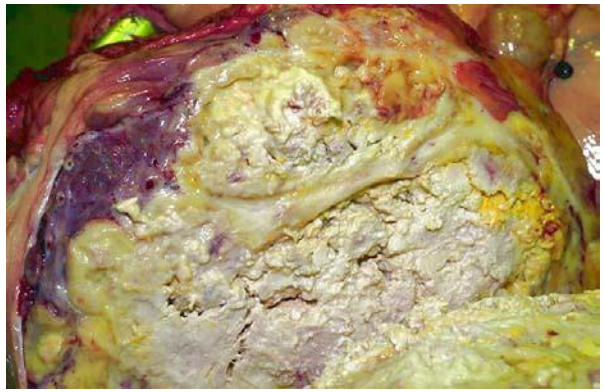
# *Rhodococcus equi* – a major horse pathogen

- Important cause of serious, sometimes fatal, lung, intestine, bone and joint infections in foals, worldwide.
- Commonly found in many horse environments and thrives in hot, dry and dusty conditions as well as in more temperate climates.
- Foals usually infected at 1-6 month of age.



Taken from Vazquez-Boland et al. 2013

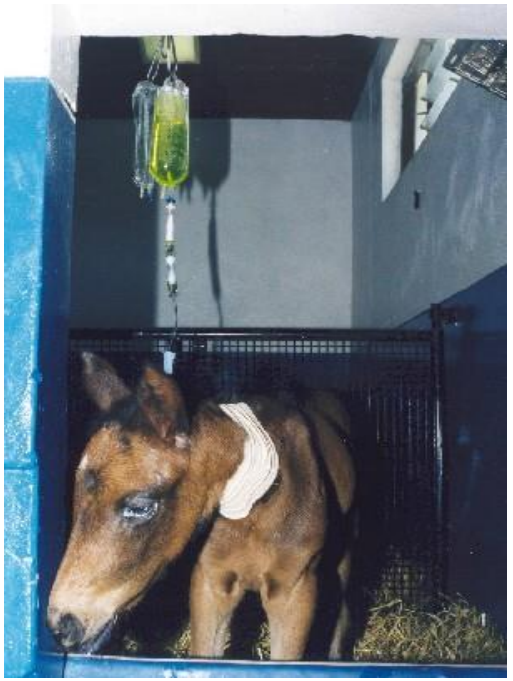




# Management of foal rhodococcosis

## Diagnosis

- Not always straight forward.
- Clinical signs suggestive but not pathognomonic.
- Blood test results (haematology and inflammatory proteins) suggestive but not pathognomonic.
- Radiographic and ultrasonographic results suggestive but not confirmative.
- Lung wash cytological results suggestive but not confirmative.
- Lung wash bacterial culture and/or PCR testing required for confirmation, ideally differentiating the VapA virulence factor.
- Accurate diagnosis required to justify use of antibiotics on basis of efficacy, foal welfare (risk of complications), encouragement of resistance and costs involved.



Courtesy Irish Equine Centre

# Management of foal rhodococcosis

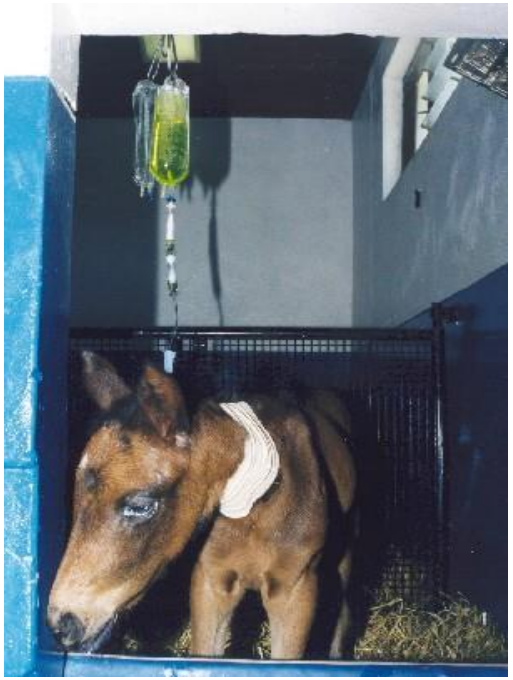
## Antibiotic therapy

- Intracellular location of the pathogen, encapsulated in pyogranulomas (chronic abscesses) causes difficult access of antimicrobial drugs to infection sites.
- Intrinsic resistance to a number of antibiotics (penicillins, cefalosporins, sulfamides, quinolones, tetracyclins, clindamycin, cloramphenicol).
- Requires combination of **rifampin and a macrolide** (erythromycin, azithromycin or clarithromycin) for a **long** (4-12 months) **and expensive course.**
- Antibiotics are sometimes used prophylactically at some endemic studs
  - questionable efficacy
  - risk of encouraging bacterial resistance
  - potential secondary effects (hyperthermia with rifampin)
  - public health implications (rifampin is a first line anti-TB drug for humans)

## Passive immunisation

- Hyperimmune horse plasma is used at some endemic studs.

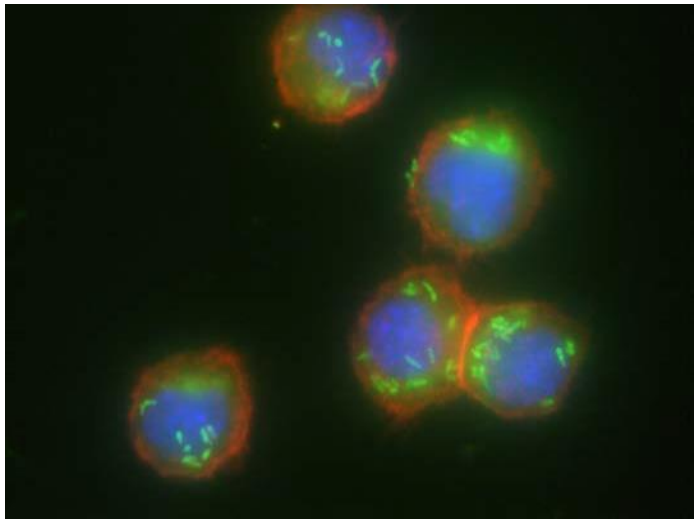
**No vaccine currently available**



Courtesy Irish Equine Centre

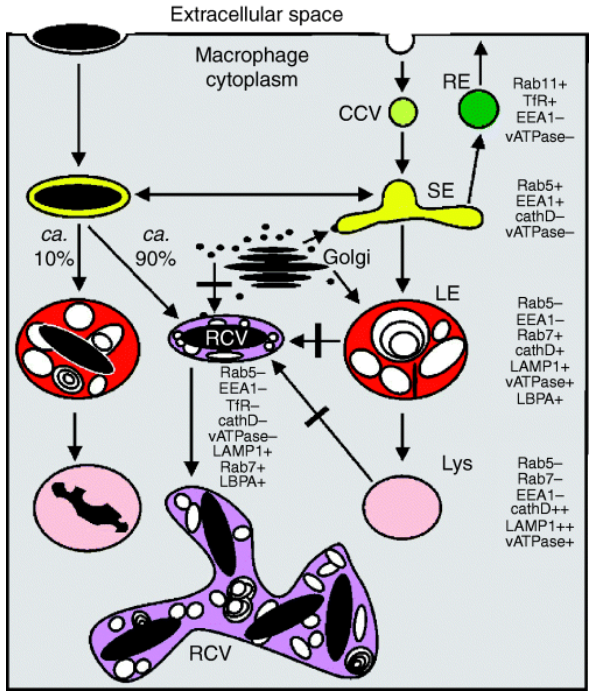
# *R. equi* – an intracellular parasite of host macrophages

- Survives and proliferates within foal macrophages (specialised blood cells that usually capture and kill infectious organisms).
- Replicates in a vacuole (Rhodococcus containing vacuole - RCV) within the macrophage (see below).
- Kills macrophages and causes local inflammatory response and tissue damage, resulting in chronic abscess formation.



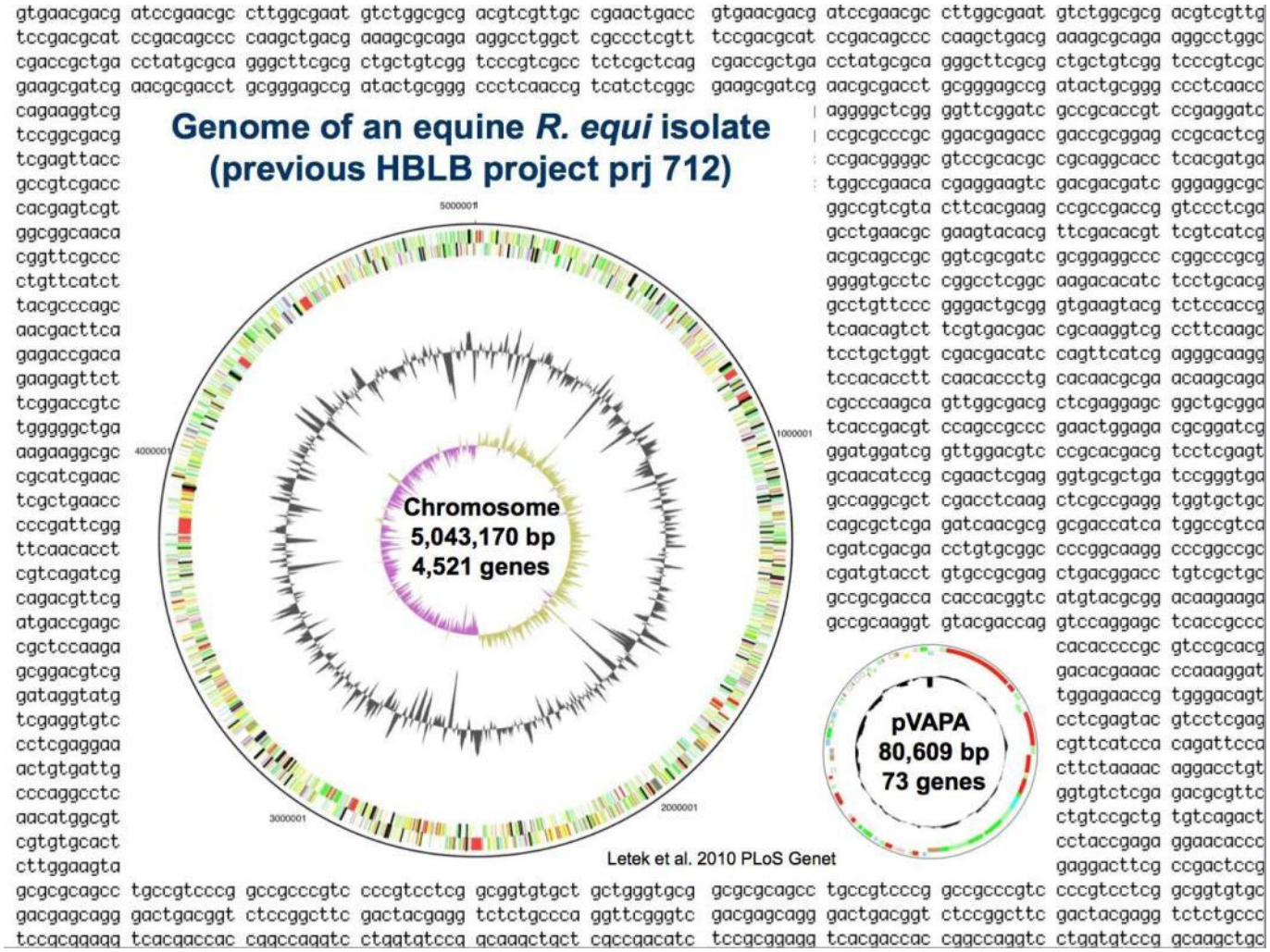
A. Hapeshi / E. Anastasi

*R. equi (fluorescing green)* in infected mouse lung infection model macrophages




Fernandez-Mora et al., Traffic 2005

# Our approach to the problem …
# Genome-based rational target identification



# Project prj 753
# Functional analysis of the *R. equi* genome

## Objectives

The previous HBLB funded project (prj 712) successfully sequenced the complete *Rhodococcus equi* genome.

Following on, this project aimed to functionally analyse the genome in order to identify:

- the organism's **mechanisms involved in pathogenic infection and transmission**, by analysis of its transcriptome (genes that are actively 'switched on' to perform important functions).
- genes with potential as **novel vaccine candidates**, by testing gene mutants (altered genes) in an innovative mouse infection model.

# Project prj 753
# Functional analysis of the *R. equi* genome

## Applicants

Professor Jose Vazquez-Boland, University of Edinburgh

Professor Wim Meijer, University College Dublin

Professor John Prescott, University of Guelph

Dr Michal Letek Polberg, University of Edinburgh

## Collaborators

Dr Ursula Fogarty, Irish Equine Centre

Dr Mariela Scortti, Complutense University of Madrid & Univ. of Edinburgh

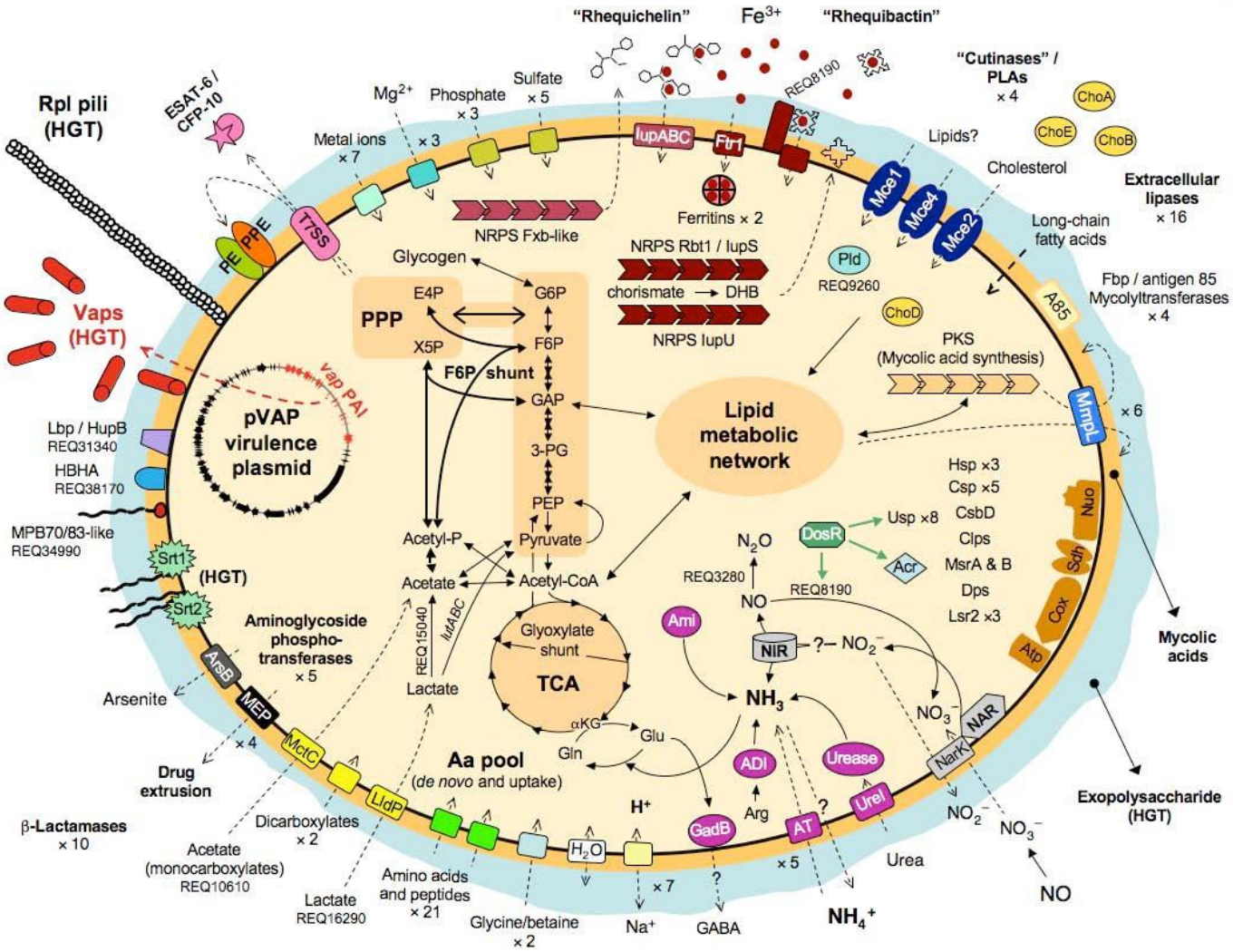
Professor Mary Hondalus University of Georgia

Dr Jolyon Holdstock Oxford Gene Technologies, Oxford Gene Technologies

Dr Andrew MacDonald, University of Edinburgh

Dr Sutherland Maciver, University of Edinburgh

# Findings - Diagramatic overview of *R. equi* based on genome analysis




Schematic overview of relevant metabolic and virulence-related features of *R. equi* 103S

See Letek et al. 2010 PLoS Genet 6:e1001145 for details

# Project prj 753
# Functional analysis of the *R. equi* genome

## Findings

*R. equi* forms long rigid appendages (pili) that form attachments to foal cells (both macrophages and epithelial cells) and these pili were found to be essential for pathogenic lung consolidation *in vivo* in the mouse lung infection model. Therefore, if *R equi* can be prevented from producing pili, it cannot infect foals.

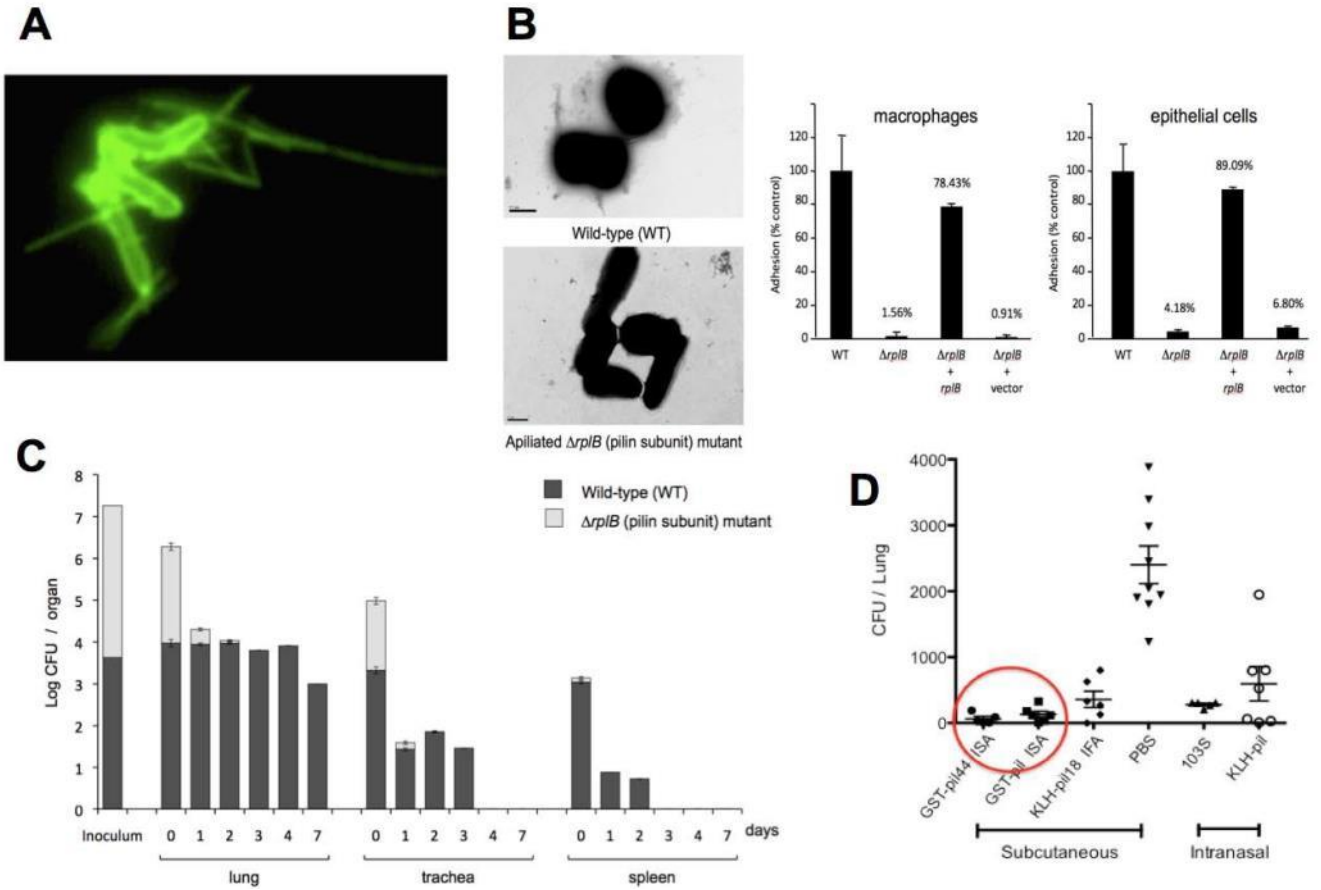
The genetic structure of the pili can stimulate production of specific antibodies that inhibit attachment of *R. equi* to the foal cell and which were found to protect mice from lung infection*, in vivo* in the infection model.

These findings have identified logical strategies for future novel vaccine design, i.e. vaccines that stimulate the production of specific anti-*R. equi* pili antibodies in foals.

These specific antibodies might produce a useful diagnostic blood test for the detection of infected foals, identifying those that require specific antibiotic treatment, early than is currently possible and, equally important, to identify those foals that do not require specific antibiotic treatment.

See the next slide for more scientific detail.

# Findings – *R. equi* cytoadhesive pili are essential virulence factors required for lung colonization and novel subunit vaccine candidate




***R. equi* pili and their role in virulence and as protective antigen**.

*(A)R. equi* pili (3-5/cell) visualised by immunofluorescence using a rabbit polyclonal antiserum.
(B)Rpl pili mediate *R. equi* attachment to macrophages (J774) and epithelial cells (HeLa), as demonstrated by *rplB* (pilin subunit) gene deletion and complementation analysis.

(C)Rpl pili are essential for lung colonization *in vivo* (murine model of *R. equi* lung infection, Balc/c mice). Competitive virulence assay in which every mouse (BALBalb/c) received an intranasal inoculum containing a 1:1 mixture of *R. equi* wild-type (WT) and in-frame *rplB* deletion mutant (Δ*rplB*) bacteria.
(D) An RplB pilin subunit experimental vaccine protects mice against an acute respiratory challenge with virulent *R. equi*

# Project prj 753
# Functional analysis of the *R. equi* genome

## Findings

*R. equi*'s capsule is linked with the organism's mucoid colonial characteristics (seen when grown in the laboratory). Non-mucoid mutant forms of the organism are significantly more susceptible to desiccation and UV radiation, conditions encountered in soil during hot dry summer weather, when transmission of *R. equi* infection regularly occurs.

*R equi'*s capsule therefore confers a competitive advantage to survival in soil and is the first 'environmental survival' factor identified to date for this pathogen.

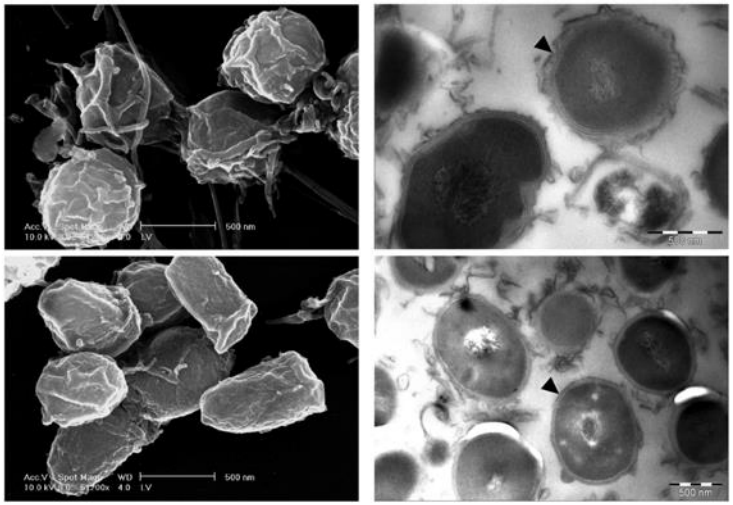
Experiments in an amoeba phagocytosis model (shown to mimic host macrophage phagocytosis) suggest that protozoa may serve as reservoirs for *R. equi* in the environment.

See the next slide for more scientific detail.

# Findings – *R. equi's* polysaccharide capsule is required for environmental survival (a transmission virulence factor)

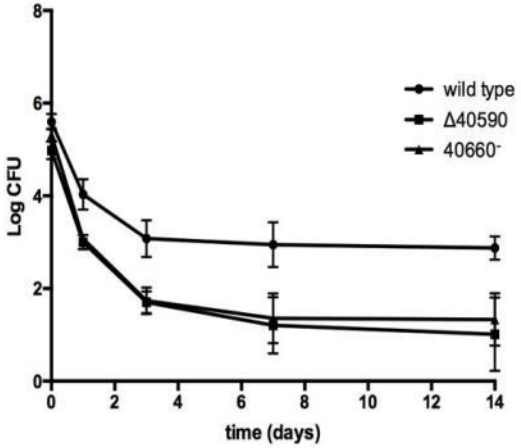
Wild type

*Δ40590*

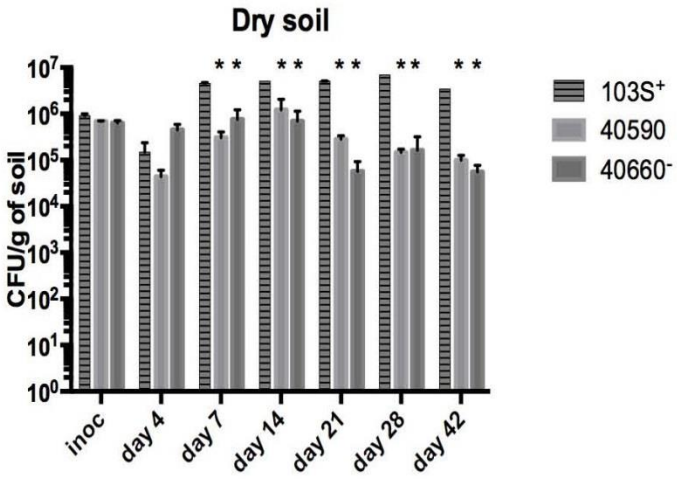


Scanning electron microscopy of *R. equi* 103S wild type and non-capsulated mutant. Note the loss of the exopolysaccharide sheath that covers *R. equi* cells.


Loss of mucoid colony phenotype in non-capsulated mutants.




Exopolysaccharide capsule protects against desiccation (left) and promotes *R. equi* survival in dry soil (right).



# Project prj 753
# Functional analysis of the *R. equi* genome

## Findings

Computer network database analysis has confirmed ‘cross-talk’ functional links between *R equi*’s virulence plasmid (VapA) and the organism’s chromosomal genome.

The main stimulus to induce R. equi’s virulence is a temperature shift from 30$^{o}$ to 37$^{o}$ (the foal’s body temperature).

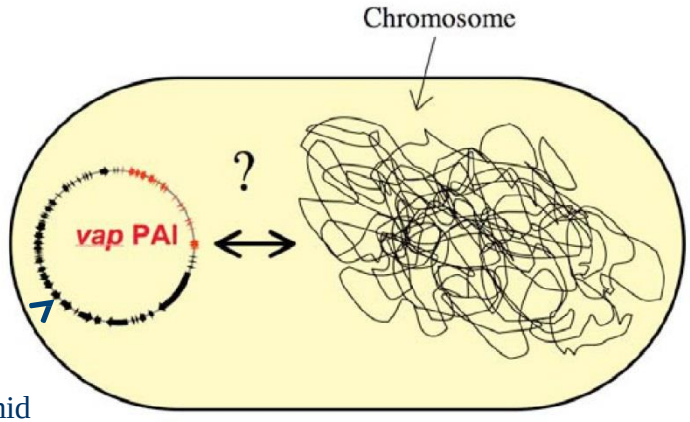
Two novel ‘metabolic virulence chromosomal genes’ were identified as candidates for future novel vaccine design.

See the next four slides for more scientific detail.

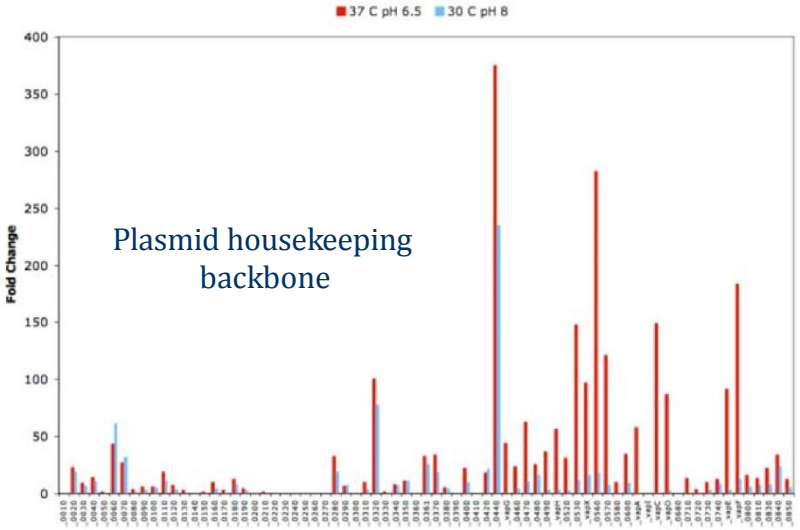
# Findings

## Identification of novel virulence genes based on their co-expression with plasmid *vap* genes

### Cross-talk between plasmid and chromosome?




Plasmid housekeeping backbone

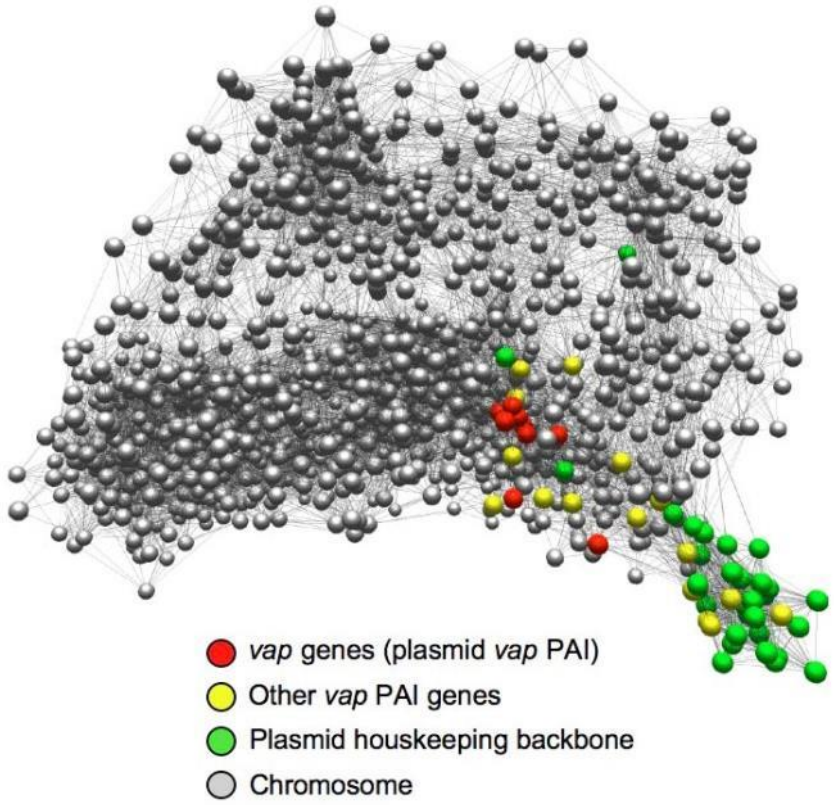



### Approach:

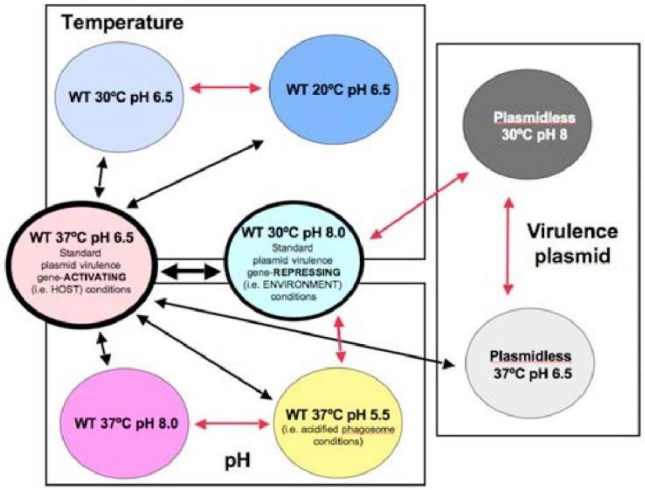
Analysis of global transcriptional response of the *R. equi* genome in the presence and absence of plasmid in conditions known to activate (37ºC-pH 6.5) or downregulate (30ºC-pH 8) the virulence genes of the plasmid *vap* PAI.

Whole-genome high-density 8×15K custom microarrays with up to four different 60-mer oligonucleotides per CDS (13,823 probes for the chromosome, 201 for the virulence plasmid) (Agilent)

# Identification of novel virulence genes based on their co-expression with plasmid *vap* genes




*R. equi* virulence plasmid genes are integrated in the chromosomal expression network



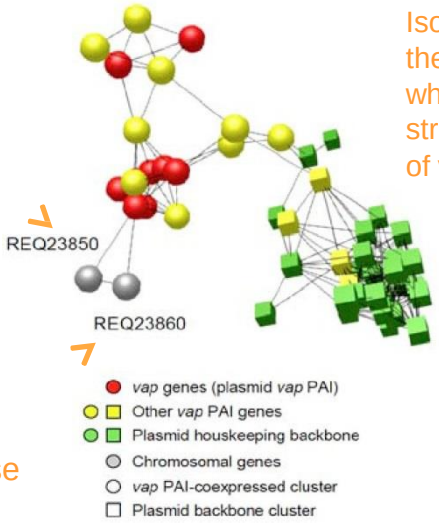

***R. equi* trancriptome network**
Each gene is represented by a node and the edges (lines) represent gene expression interrelationships; the closer the nodes sit in the network the stronger the correlation in their expression profile.

Rather than performing classical pairwise comparisons between the different test conditions (specified above in the inset on the right), we analyzed the expression data globally using a network approach. This identified functional connections between the virulence plasmid and a number of chromosomal genes (which consequently were selected as candidate virulence genes for analysis).

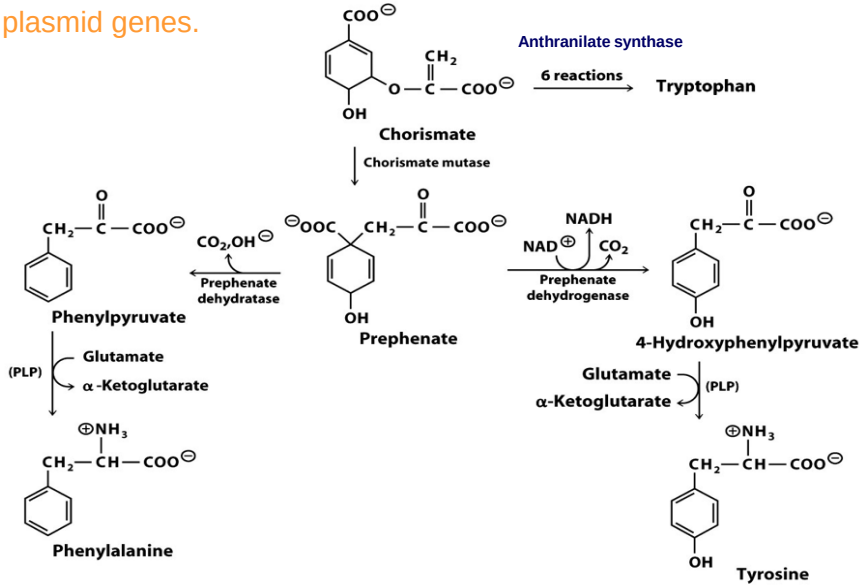
# Transcription network analysis identifies two novel 'metabolic virulence genes' involved in aromatic amino acid biosynthesis

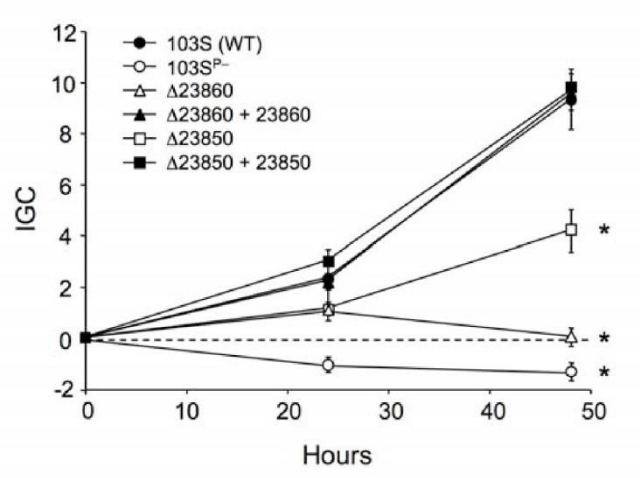
AroQ (type II) chorismate mutase

TrpEG bifunctional anthranilate synthase




Isolated subnetwork showing the two chromosomal genes who expression is most strongly correlated with that of virulence plasmid genes.



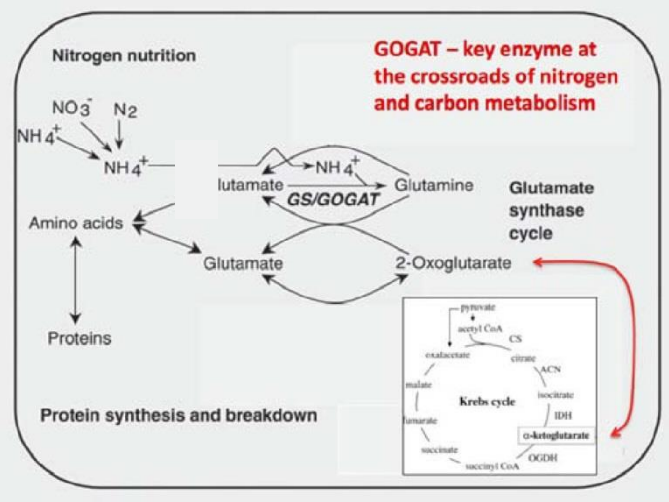




The virulence plasmid-coregulated chorismate mutase REQ23850 and anthranilate synthase REQ23860 are required for efficient replication in macrophages.

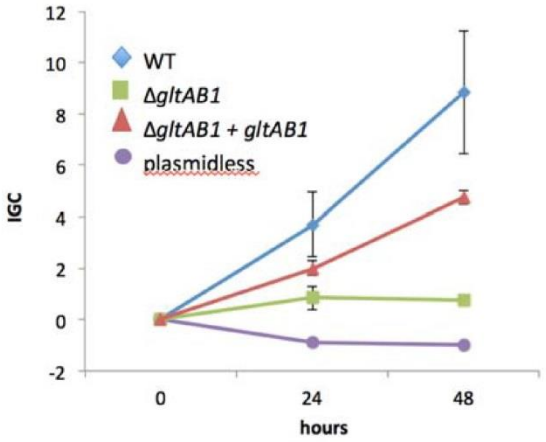
These enzymes catalize the first committed steps in aromatic amino acid biosynthesis, implying that the vacuole in which *R. equi* replicates within macrophages is poor in aromatic aminoacids.

# Glutamate synthase GltAB1 is also co-regulated with plasmid virulence genes: novel key metabolic virulence factor and target for attenuated vaccine

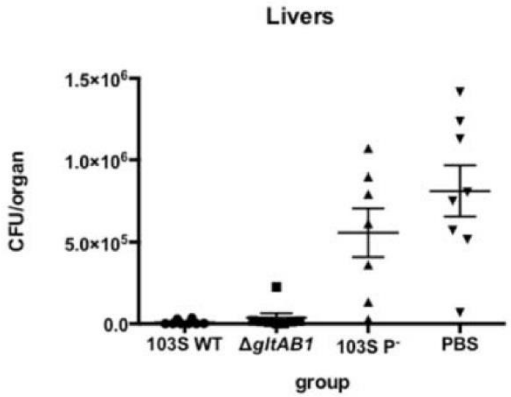
| | | | | | | | |
|---|---|---|---|---|---|---|---|
| REQ1810 | 182545 | 183033 | class=3.4.4 colour=6 | putative thioesterase | 0.031376675 | 2.3665643 | up |
| REQ35090 | 3727451 | 3728020 | class=3.4.3 colour=6 | putative aromatic ring-opening dioxygenase | 0.004533808 | 2.2387245 | up |
| REQ35040 | 3722886 | 3723320 | class=3.4.4 colour=6 | putative thioesterase | 0.035759225 | 2.2007816 | up |
| REQ35200 | 3736985 | 3737917 | class=3.4.3 colour=6 | catechol 2,3-dioxygenase | 0.012968476 | 2.0051382 | up |
| REQ0450 | 51042 | 52493 | class=3.1.7 colour=7 | glutamate synthase small subunit GltB1 | 0.009359228 | 33.846283 | up |
| REQ0440 | 46463 | 51049 | class=3.1.7 colour=7 | glutamate synthase large subunit GltA1 | 0.017122857 | 11.234403 | up |
| REQ42020 | 4467404 | 4468597 | class=3.1.0 colour=7 | imidazolonepropionase | 0.00347291 | 8.361309 | up |
| REQ41910 | 4451894 | 4455313 | class=3.1.7 colour=7 | putative glutamate dehydrogenase | 0.005696159 | 8.236173 | up |
| REQ4890 | 513483 | 514859 | class=3.0.0 colour=7 | putative oxidoreductase | 0.002848462 | 8.144812 | up |
| REQ41950 | 4458761 | 4459909 | class=3.0.0 colour=7 | putative CoA-transferase | 0.01222926 | 7.0029726 | up |
| REQ40650 | 4328471 | 4329814 | class=3.3.1 8 colour=7 | UDP-glucose 6-dehydrogenase | 0.000500973 | 6.464989 | up |
| REQ15250 | 1590586 | 1592904 | class=3.0.0 colour=7 | putative pyruvate water dikinase | 0.006347012 | 6.2924213 | up |




REQ0440 (*gltA1*) and 0450 (*gltB1*) encode the large and small subunits, respectively, of an NADPH-dependent glutamate synthase (GOGAT). Key role in nitrogen assimmilation and de novo amino acid biosynthesis.

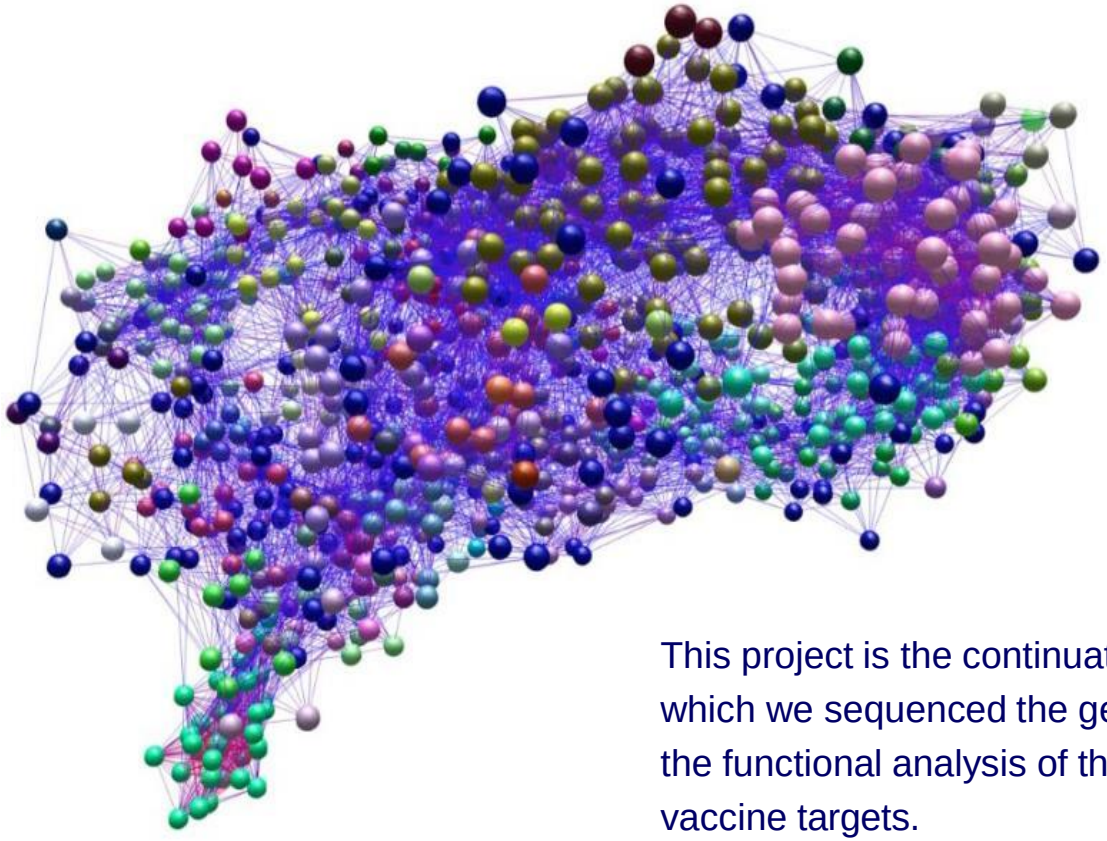



Impaired intracellular survival of *gltAB1* mutant in macrophages. Mutant also strongly attenuated in mice.




Efficacy of *gltAB1* attenuated mutant as an attenuated vaccine in mice

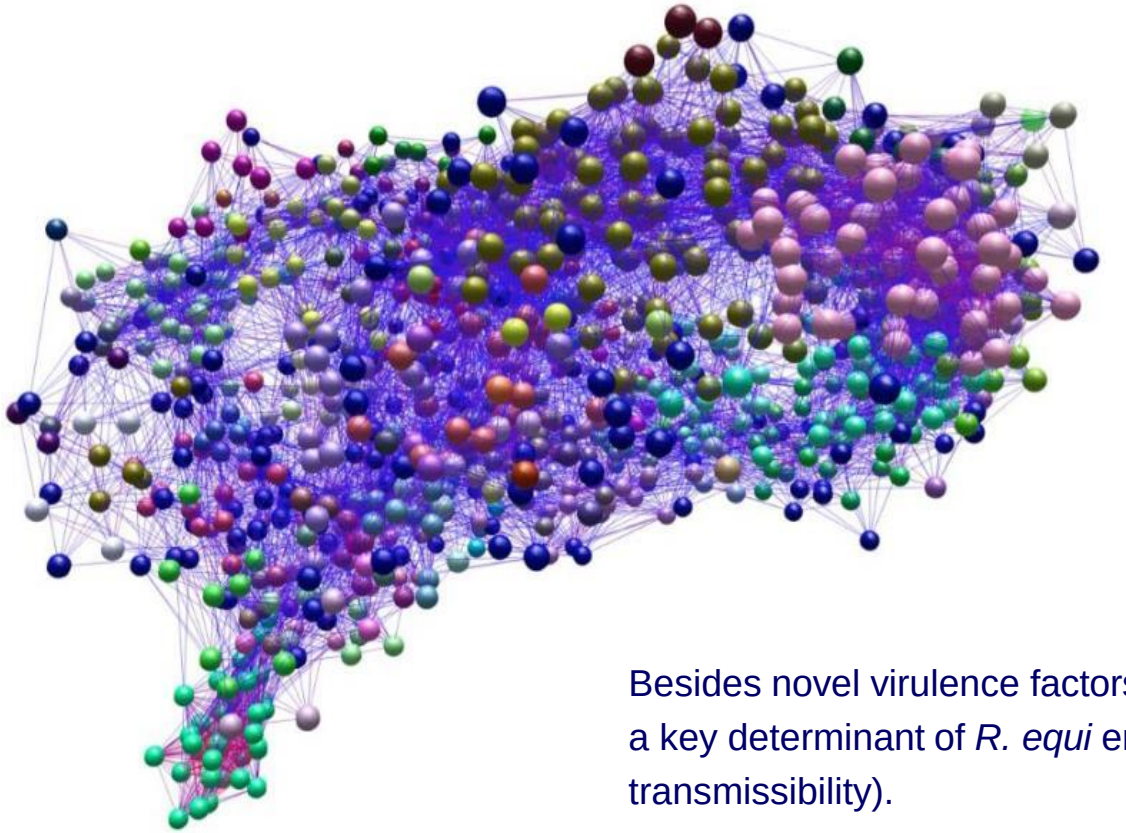
# Summary, conclusions & impact



This project is the continuation of a previous HBLB-funded project in which we sequenced the genome of *R. equi*. Here we have undertaken the functional analysis of the genome in our quest to identify novel vaccine targets.

We identified two novel major virulence determinants of *R. equi*, the cytoadhesive pili and important enzymes. For these we demonstrated, using a novel *in vivo* lung infection model in mice, their potential as *R. equi* vaccine candidates.

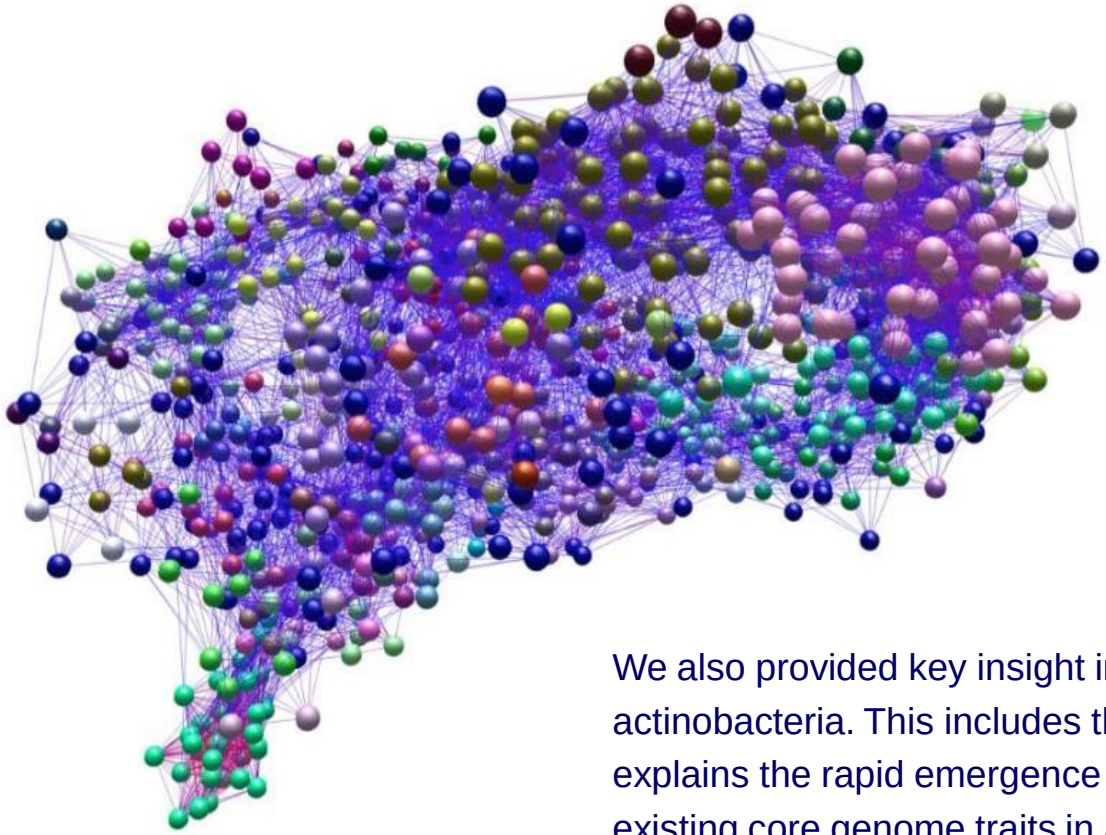
# Summary, conclusions & impact



Besides novel virulence factors, we identified, in the organism's capsule, a key determinant of *R. equi* environmental survival (and hence transmissibility).

This project has significantly advanced our knowledge of the biology and pathogenesis of this important horse pathogen.

# Summary, conclusions & impact



We also provided key insight into the evolution of virulence in pathogenic actinobacteria. This includes the notion of ‘cooptive virulence’, which explains the rapid emergence of pathogenicity via the ‘appropriation’ of existing core genome traits in a process triggered or accelerated by a few decisive host-adaptive horizontal gene acquisitions. Most of the co-opted virulence genes identified in *R. equi* are housekeeping metabolic genes, providing key insight into the metabolic determinants of *R. equi* pathogenesis and the nutritional composition of the within-host habitat in which this microrganism survives and replicates.