


Genetic analysis of *Salmonella* & *E. coli* pathogenesis in farm animals  
Mark Stevens, Institute for Animal Health

**EADGENE** European Animal Disease Genomics Network of Excellence for Animal Health and Food Safety

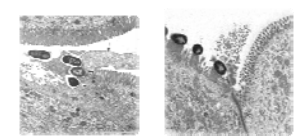
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*Salmonella enterica* & *Escherichia coli*

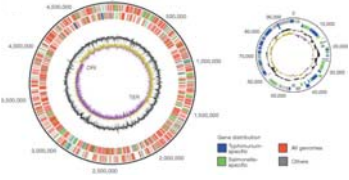


- Zoonotic diarrhoeal pathogens of worldwide importance
- Also produce morbidity & mortality in food-producing animals
- Presentation varies from subacute, enteritis to severe systemic disease depending on serotype-, pathotype- & host-specific factors
- Evolution punctuated by emergence of epidemic & MDR variants
- Improved strategies required to control animal & zoonotic infection

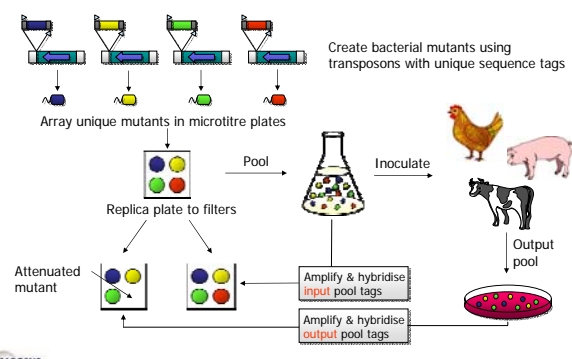
Stevens et al. 2009. *Philos Trans R Soc Lond B Biol Sci.* 364:2709-23;  
Dziva & Stevens. 2009. *Avian Pathol.* 37:355-66; Stevens & Wallis. 2005. *EcoSal* 8.3.3.2. ASM Press

Genome sequences provide powerful insights into the evolution of *Salmonella* & *E. coli* virulence, but..

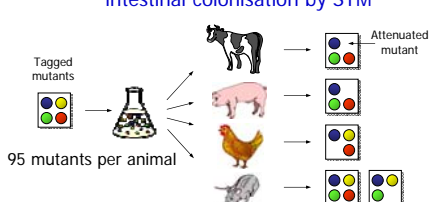
- What role do the genes play in colonisation, pathology & transmission?
- When, where and at what level are they expressed?
- What is the impact of variation in repertoire, sequence or expression?
- What determines host range and zoonotic/epidemic potential?
- How do the encoded proteins work?
- Which genes encode protective antigens?



Identification of bacterial virulence factors by signature-tagged transposon mutagenesis (STM)



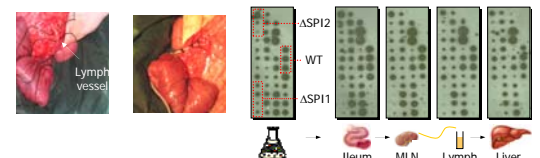
Identification of *S. Typhimurium* genes mediating intestinal colonisation by STM



- 1045 *S. Typhimurium* mutants screened in calves, pigs, chickens & mice
- 227 attenuated mutants identified
- Factors playing conserved roles identified (e.g. T3SS-1 & -2)
- Host-specific virulence factors identified (e.g. SPI-4-encoded T1SS)
- Mice often failed to detect factors needed in livestock

Morgan et al. 2004. *Mol. Microbiol.* 54:994-1010; Carnell et al. 2007. *Microbiol.* 153:1940-52.

Tagging methods also reveal the temporal & spatial role of *Salmonella* genes in the pathogenesis of typhoid

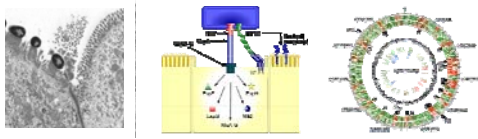


- Surgical cannulation used to define the mode & genetics of systemic translocation of *S. Dublin* in cattle
- Visceral organs seeded by clones migrating via lymphatics, not blood
- SPI-1 (active uptake), but not SPIs 2-5 & other virulence-associated loci vital for this process
- *S. Dublin*-specific island (SDI-1) identified by subtraction also required
- Temporal & spatial role of 30 sensory systems evaluated

Pullinger et al. 2007. *Infect. Immun.* 75:5191-9; Pullinger et al. 2008. *Infect. Immun.* 76:5310-21

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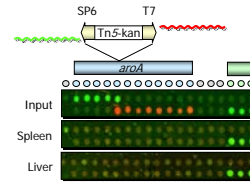
Identification of enterohaemorrhagic *E. coli* (EHEC) genes mediating colonisation of cattle by STM



- 1900 EHEC O157 & 570 EHEC O26 mutants screened in calves
- >120 factors mediating intestinal colonisation, inc. conserved & serotype-specific factors
- T3SS, intimin-Tir interactions & selected effectors play key roles
- Accessory roles defined for cytotoxins (LifA, EspP) & F9 fimbriae
- Intimin-, Efa1- & EspA-based vaccines tested in cattle

EADGENE Dziva et al. 2004. Microbiol. 150:3631; Stevens et al. 2002. I&I 70:5158; van Diemen et al. 2005. I&I 73:1735; Low et al. 2006. I&I 74:2233; van Diemen et al. 2007. Vet. Imm. Imm. 116:47

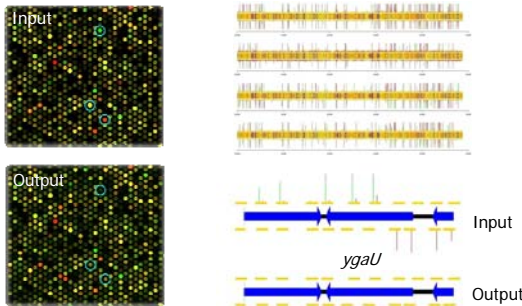
Global assignment of the role of *Salmonella* genes in livestock by transposon-mediated differential hybridisation



- Genome mutated using a transposon with divergent phage promoters
- Run-off transcripts hybridised to a high-density oligonucleotide array
- Simultaneously maps insertion site & yields a numerical attenuation score for each transposed gene
- Phenotypes are derived for all [mapped] mutations (cf. STM)

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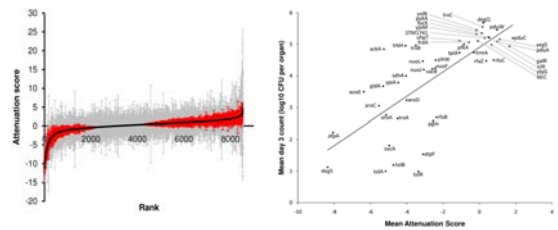
The location & attenuation index of hundreds of mutations can be simultaneously evaluated



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Application of TMDH in a murine typhoid model

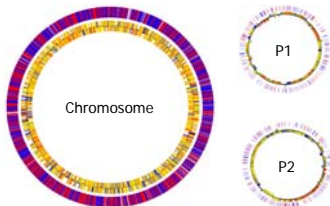
- 5184 Tn5 & 5184 Mu SL1344 mutants screened in BALB/c mice (480/pool 10<sup>6</sup> IV with recovery from liver & spleen 2 DPI)
- 6108 unambiguously mapped to 2824 different *S. Typhimurium* genes
- Good correlation between TMDH attenuation score & recovery of 47 defined mutants after single infections



Chaudhuri RR et al. 2009. PLoS Pathog. 5:e1000529 UNIVERSITY OF CAMBRIDGE

Creation & screening of a mutant library of *S. Typhimurium* 4/74

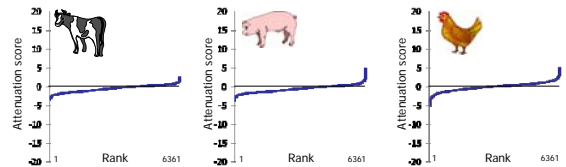
- SL1344 proved attenuated relative to the parent (ST4/74) in farm species
- ST4/74 electroporated with transposomes using custom Tn for TMDH
- Of 8550 mutants, insertion sites mapped for 6604 in c. 3200 genes:



- 8550 mutants screened by oral inoculation of calves, pigs & chickens (475/calf or pig; 95/duplicate chickens)

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The majority of random mutations do not exert a significant attenuating effect in food-producing animals

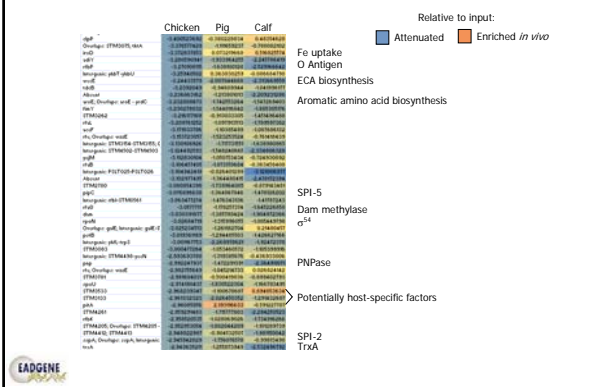


- No evidence of high-frequency random loss at the pool complexities selected
- Good correlation between repeated pools
- Recoveries of >10<sup>4</sup> colonies required for reliable scoring

# Genetic analysis of Salmonella & E. coli pathogenesis in farm animals

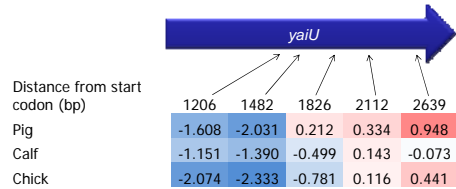
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## TMDH provides key data on the role of thousands of *S. Typhimurium* genes in reservoir hosts



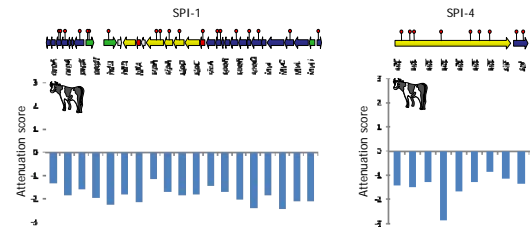
## TMDH can yield functional annotation at the intragenic level

Presence of multiple transposon insertions within the same gene reveals positional effects of mutations



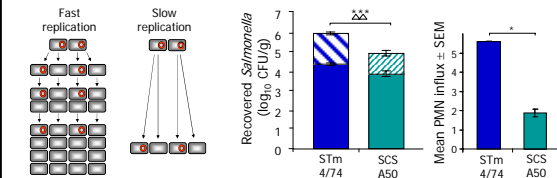
## Correlation of STM & TMDH findings

- Of attenuating mutations identified in calves by STM, comparable inserts were found for 75 mutants in the TMDH library
- 73.3% of similar mutations were negatively selected by both methods at an attenuation score of  $\leq -1.0$  (84% at  $\leq -0.5$ )



## Integrating gene function with other datasets to unravel the differential virulence of *S. enterica* serovars

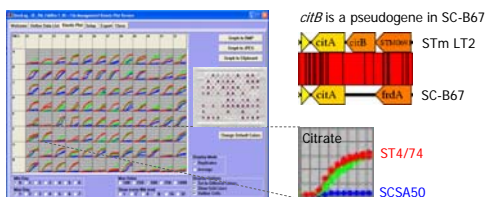
- In pigs *S. Choleraesuis* SCSA50 causes typhoid & mild enteritis but *S. Typhimurium* ST4/74 elicits acute enteritis and is cleared
- Plasmid partitioning indicates that ST4/74 replicates faster than SCSA50 in porcine intestinal mucosa
- Associated with elevated induction of IL-8, IL-18 & TNF $\alpha$  mRNA & <sup>111</sup>In-neutrophil influx



Watson et al. 2000. Microbiol. 146:1639-49; Paulin et al. 2007. Infect. Immun. 75:3950-60

## Genome sequencing & phenotype microarrays reveal differences in metabolic functions between serovars

- S. Choleraesuis* SC-B67 has pseudogenes in many metabolism-related genes compared to *S. Typhimurium* LT2 (Chiu et al. 2005. NAR 33:1690-8)
- PM array analysis of 960 phenotypes reveals differences in metabolic activity, e.g. ST4/74, but not SCSA50, respire citrate



## Multiple *S. Typhimurium cit* mutants were negatively selected in the porcine intestines by TMDH



Signature-tagged mutagenesis in pigs identified an attenuating mutation in *citC*

So far TMDH in pigs has found attenuating mutations in:

- citA-B*
- citB*
- citC*
- citC2-D2*
- citD*
- citF2*
- citG2*
- citI*
- citX*

- Example of a polymorphic system required for pig colonisation by *Salmonella* that could explain the different net replication rate of serovars
- A systems approach is needed to integrate & interrogate datasets

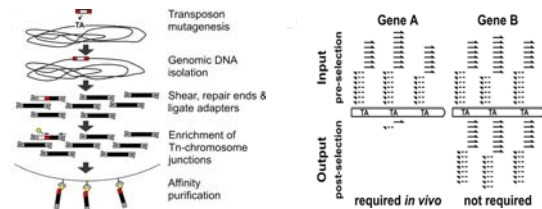
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## Outlook for the future...

The advent of massively parallel sequencing permits analysis of mutant fitness in complex pools by sequencing of Tn ends

- Tn-Seq [van Opijnen et al. 2009. Nat. Meth. 6:767-72](#)
- HITS [Gawronski et al. 2009. PNAS 106:16422-7](#)
- TRaDIS [Langridge et al. 2009. Gen. Res. In press](#)



## Concluding remarks

- Functional annotation of pathogen genomes is a major challenge of the post-genomic era
- Serotype-, host- & niche-specific virulence factors exist & studies in target hosts are required
- Use of strains of well-defined virulence in animals is required to formally link genotype-to-phenotype
- Genome-wide mutagenesis reveals powerful insights into the temporal & spatial role of *Salmonella* & *E. coli* genes
- New methods offer higher resolution but can be complicated by population dynamics
- Studies with non-polar mutant & repaired strains remain important
- Key data for 'systems' approaches & rational design of control strategies

## Acknowledgements

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