

Food-borne Pathogens, Functional Genomics and Farm Animals

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Bacterial Infection Group Research Programmes

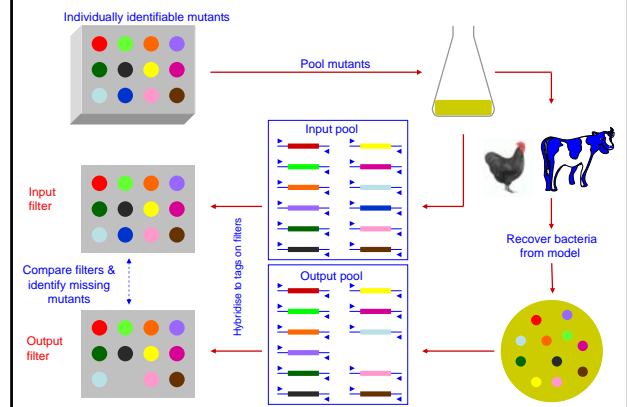
Virulence and immunity research in a range of hosts into:

- *Salmonella*
- *Campylobacter*
- *Streptococcus*
- *Staphylococcus*
- *Bordetella*
- *Haemophilus parasuis*
- *Mannheimia haemolytica*
- All underpinned by Molecular Biology, Functional Genomics, Cell Biology and Immunology

Functional Genomics Technologies

- **Signature-tagged mutagenesis (STM)**
 - powerful parallel technique
 - reduces animal usage
 - labour intensive
 - misses certain important virulence determinants e.g. secreted toxins
- **Microarray expression technology**
 - Technically complex, especially for *in vivo* applications
 - Controls for comparison experiments conceptually difficult
 - Interpretation difficult

Signature Tagged Mutagenesis



Farm Animal STM Work

- *S. typhimurium* STM in calves, chickens and pigs
- *C. jejuni* STM in chickens for colonisation
- STM in veterinary streptococci in organ culture, pigs

STM in *Salmonella* in calves and chicks

- To compare the same pools of mutants in a calf model and a chicken model of infection
- A direct attempt to pin down some host specificity genes and to compare the requirements for survival in the two hosts

STM in *Salmonella* in calves and chicks

- Results show that different sets of genes are required in the different hosts.
- For infection of calves more of the “virulence” genes needed, (e.g. *Salmonella* pathogenicity islands or Spi), whereas for chickens more metabolic genes

Calf vs chick:

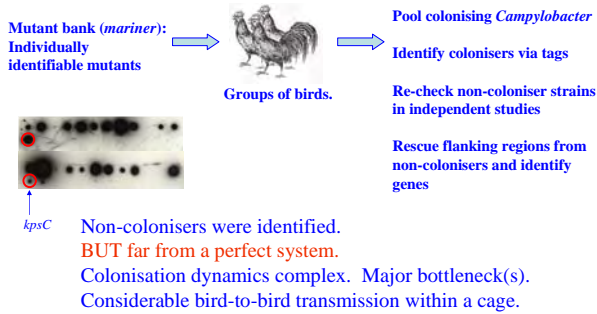
Spi-1 16 vs 2, Spi-2 16 vs 1, Spi-3 4 vs 2, Spi-4 3 vs 0

Metabolic+Regulatory+Hypotheticals 40 vs 65 with much overlap

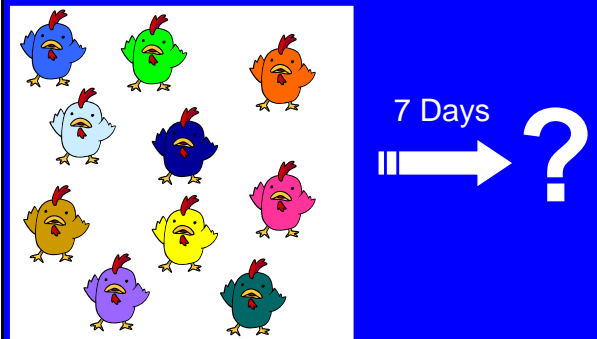
***Campylobacter jejuni* STM**

- *In vitro* mutagenesis system using modified mariner transposon developed, incorporating plasmid rescue from mutants of interest
- Screening of mutant pools through 2-week old chick model

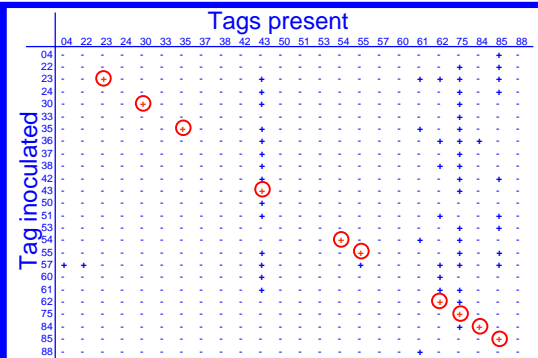
***Campylobacter jejuni* STM**



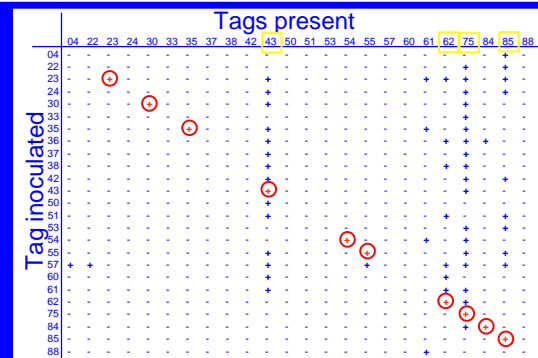
Inoculate each bird with a single mutant, house in a pen together – identify colonising mutants after 7 days



Presence of tags in individual birds day 7 p.i. M1 tagged mutants



Presence of tags in individual birds day 7 p.i. M1 tagged mutants



Do these dominating mutants have a colonisation advantage, have they adapted *in vivo*, or is it chance?

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Tag	Insert site	Predicted function	Motility
04	ND (bad tag)		Y
22	gj1095	Integral membrane protein, weak similarity to <i>E. coli</i> acetyltransferase	NG plate
23	gj1506c	Probable MCP-type signal transduction protein	Y plate, N microscope
24	gj1334	Unknown	ND
30	ND (in progress)		Y
33	gj1528	Pseudogene in 11168 - CJ1528, probable transmembrane transport protein similar to <i>E. coli</i> C4-dicarboxylate anabolic carrier	Y
35	gj0988c	Questionable CDS	Y
36	gj1194	Possible phosphatase permease	N
37	176.83	<i>C. jejuni</i> CPS locus, Unpublished	<wt plate, N microscope
38	gj0337c	<i>motA</i> , flagella motor protein	N
42	gj0637c7	Possible ribonuclease (22 bp sequence available)	Y
43	gj0970	Unknown	NG plate, N microscope
50	gj1471c	Probable type II secretion system E protein (channel protein, SecYEG complex)	Y
51	gj0013	<i>ivd</i> , probable dihydroxy-acid dehydratase	<wt plate, < wt microscope
53	gj1544c	Probable integral membrane protein of unknown function	<wt plate, Y microscope
54	gj0949c	Possible transmembrane transport protein	NG plate, N microscope
55	111828	<i>C. jejuni</i> 11828 LOS locus (AF343914), CDS of unknown function	Y
57	gj1716c	<i>ivuD</i> , probable 3-isopropylmalate dehydratase small subunit	ND
60	gj0959c	<i>rpsA</i> , probable 30S ribosomal protein S1	Y
61	M1 unique?	No matches in GenEMBL	Y
62	gj1633	Unknown (heme binding signature)	Y
75	M1 unique?	No matches in GenEMBL	Y
84	gj1469	Probable integral membrane protein	Y
85	gj1256c	Possible membrane protein, no <i>Hp</i> match	ND
88	gj1565c	<i>pilA</i> , required for flagellar function	N

Mutant	Prevalence	Insert site / Predicted function
75-04	17/25	M1 unique?
43-04	14/25	gj0970: 100aa, no GenEMBL matches
85-04	9/25	gj1256c: possible membrane protein, no <i>Hp</i> match
62-04	8/25	gj1633: heme-binding signature, <i>Hp</i> match

Bird-to-bird transmission & irreproducibility between experiments in individually inoculated, mixed cage colonisation experiments

Experiment 1						Experiment 2					
Tag number	22	23	24	33	42	Tag number	22	23	24	33	42
Bird 1 input	X					Bird 1 input	X				
Bird 1 output	X	x	x	x	x	Bird 1 output	X	x	x	x	x
Bird 2 input		X				Bird 2 input		X			
Bird 2 output	X	x	x	X	x	Bird 2 output	x	x	X	x	x
Bird 3 input				X		Bird 3 input			X		
Bird 3 output	X	x	X	x	x	Bird 3 output	x	x	X	x	x
Bird 4 input					X	Bird 4 input				X	
Bird 4 output	X	x	x	x	x	Bird 4 output	x	x	x	X	x
Bird 5 input					X	Bird 5 input				X	
Bird 5 output	X	x	x	x	x	Bird 5 output	x	x	X	x	x

Each of five birds was individually infected with 10^8 c.f.u. of a different *C. jejuni* M1 STM. Birds were housed in a single cage and presence of tags in the caecum of individual birds assessed at day seven post infection.

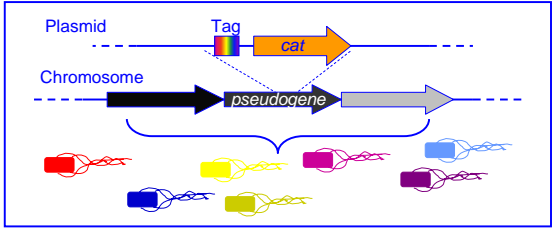
Problems encountered in applying STM to study the colonisation of the chicken caeca by *C. jejuni*

- We have identified a few mutants defective for colonisation – *kpsM*, *rpoN*
- We observe a high frequency of mutant loss from the birds suggesting that there is a bottleneck in colony establishment
- Instead of one bird/many mutants, if we inoculate a number of birds with single mutants, we see “random” between-bird transmission
- Despite an initial inoculum of each mutant at 10^8 c.f.u./ml, some mutants can displace others from a bird within a few days—competitive exclusion?

We hypothesise that a bottleneck in the colonisation process and/or bird-to-bird transmission of *Campylobacter* leads to random loss of mutants

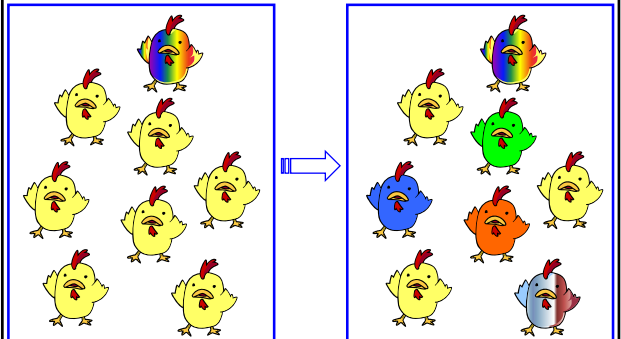
Can we address this ?

Create a number of ‘wild-type’ isogenic tagged *C. jejuni* strains (WITS), to enable us to study *C. jejuni* colonisation in the chicken and also the spread of *C. jejuni* in the environment



Monitoring passage of *C. jejuni* through a flock

Inoculate one bird with *n* tags, introduce into a pen with ‘clean’ birds. PM/swab birds on days 1, 2, 3 etc... to monitor spread of *campylobacter*

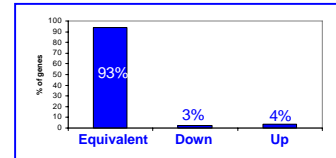
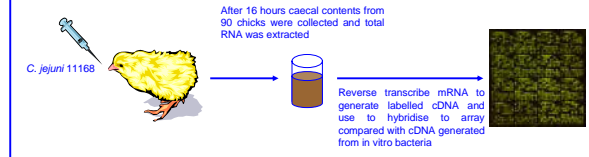


- Results so far are very complex, even with only two tags, but are completely in line with results from the STM screen
- We can “count” the relative representation of the different tagged strains in the chicken very accurately by qPCR.
- Mathematical modellers are looking at the data
- There are bottlenecks and adaptation of the bacteria for improved colonisation of the gut
- Is the concept of colonisation even meaningful with this bacterium?

Microarrays

- DNA-DNA relatedness
 - Uses in strain identification
 - Molecular epidemiology
 - “Master” arrays
- Expression profiling
 - Experiments *in vitro*
 - e.g. heat-shock, pH, other stressors
- Reliable data generated *in vivo*
 - Cell culture models for *Salmonella*
 - Data obtained from *Campylobacter* straight from chicken caeca

Microarray analysis of *C. jejuni* gene expression in the chicken host



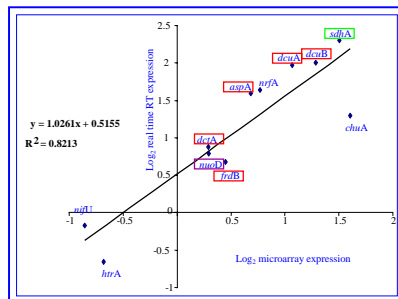
Genes up-regulated in the chick caecum compared to *in vitro* controls

Class	Gene designation	Function	Fold Change	P-value
Small molecule metabolism				
Deamination	cj1624c	L-serine dehydratase, sdaA	3.10	1.78 x 10 ⁻⁴
Energy metabolism				
Tricarboxylic acid cycle	cj0437	Succinate dehydrogenase, sdhA	32.11	1.23 x 10 ⁻¹⁴
	cj0438	Succinate dehydrogenase, sdhB	31.25	<0.05
	cj0439	Succinate dehydrogenase, sdhC	15.65	5.18 x 10 ⁻⁴
	cj0450	Putative fumarate reductase, frbB	2.51	<0.05
	cj0410	Putative fumarate reductase, frbB	2.83	<0.05
Electron Transport				
	cj1487c	Cytochrome C oxidase, cooP	3.78	2.20 x 10 ⁻⁴
	cj1488c	Cytochrome C oxidase, cooD	3.16	<0.05
	cj1489c	Cytochrome C oxidase, cooD	4.18	2.37 x 10 ⁻⁴
	cj1490c	Cytochrome C oxidase, cooD	4.40	1.30 x 10 ⁻⁴
	cj0780	Probable ferredoxin, nraA	2.87	<0.05
	cj0781	Probable ferredoxin, nraD	4.26	7.34 x 10 ⁻¹¹
	cj0783	Probable ferredoxin, nraB	2.72	<0.05
	cj1184c	Ubiquinol cytochrome C reductase, pufC	5.69	5.07 x 10 ⁻⁴
	cj1185c	Ubiquinol cytochrome C reductase, pufB	5.43	4.50 x 10 ⁻⁴
	cj1186c	Ubiquinol cytochrome C reductase, pufK	4.73	2.98 x 10 ⁻⁴
	cj1357c	Putative periplasmic cytochrome C, cvrK	5.88	1.90 x 10 ⁻⁴
	cj1358c	Probable ferredoxin, nraC	3.77	<0.05
Central intermediary metabolism				
General	cj0087	Aspartate ammonia lyase, aspA	4.83	<0.05
Signal transduction	cj0448c	Probable MCP protein, acfB	3.09	1.21 x 10 ⁻⁴
Cell processes				
Transport and binding proteins	cj1614	Haematin uptake outer membrane protein, chuA	48.08	6.25 x 10 ⁻¹⁴
	cj1615	Haematin uptake permease, chuB	4.07	<0.05
	cj1619	Probable alpha-ketoglutarate permease, kgtP	2.92	1.83 x 10 ⁻⁴
	cj0389	Anaerobic C ₄ -dicarboxylate transporter, dcbA	6.63	6.83 x 10 ⁻⁴
	cj0671	Anaerobic C ₄ -dicarboxylate transporter, dcbB	19.24	3.25 x 10 ⁻⁴
Detoxification	cj0398	Putative cytochrome peroxidase c-651, cypR	11.71	9.77 x 10 ⁻⁴
Miscellaneous	cj0833c	Probable isodroductase, yfjC	4.83	6.92 x 10 ⁻⁴

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Regression analysis showing the correlation between microarray and real time RT-PCR



Microarray analysis of *C. jejuni* gene expression in the chicken host

Up-regulated genes indicate adaptation in the caecum and include:

- Those involved in energy conversion/production
- The C₄-dicarboxylate uptake and transport system (suggesting that electron acceptors for respiration are probably C₄-dicarboxylates)
- Serine dehydratase, implying a requirement for serine
- Genes required for growth/survival in reduced oxygen
- Activation of an oxygen requiring succinate dehydrogenase operon
- The number of *C. jejuni* genes differentially expressed when the bacterium is growing in the chicken compared with *in vitro* is small

Are any of these VACCINE TARGETS?

Maybe as components of a combined *Salmonella/Campylobacter* vaccine?

Transposon Mutants + Microarrays: Transposon Mediated Differential Hybridisation (TMDH)

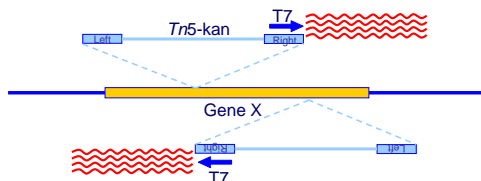
A protocol that :

- enables the simultaneous analysis of thousands of bacterial transposon mutants using whole genome micro-arrays
- identifies putative essential genes in bacterial pathogens that may be targets for anti-microbial drug discovery

The Five Components of TMDH

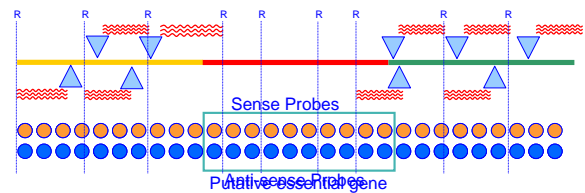
1. A sequenced bacterial genome
2. A randomly integrating transposon or a number of transposons with different integration preferences
3. The ability to generate labelled DNA/RNA flanking the site of transposition (known as target)
4. A good quality whole genome array (probes).
5. Novel bioinformatics

Transposons for TMDH

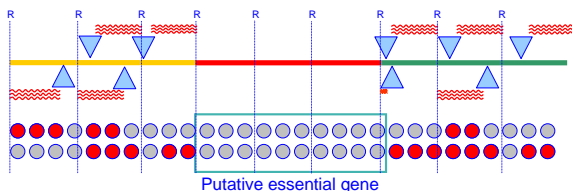


- Transposition leads to incorporation of RNA polymerase promoters at integration sites
- *In vitro* transcription reactions on purified chromosomal DNA generates labeled target specific to the point of integration that can be analysed via an array

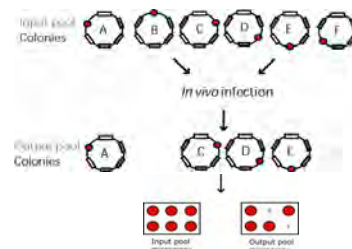
Schematic result



Schematic result



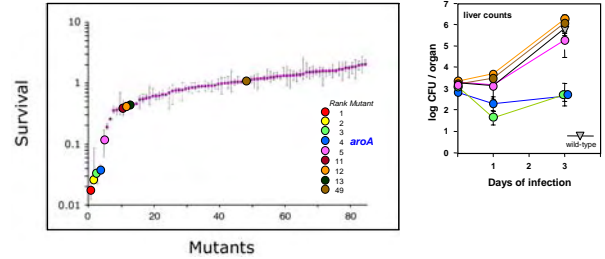
In vivo TMDH



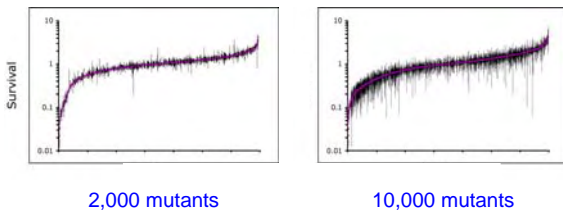
TMDH versus STM

1. Faster
2. More accurate
3. Complete information
4. (Semi-)Quantitative
5. Easier (less labour intensive)
6. More mutants per animal

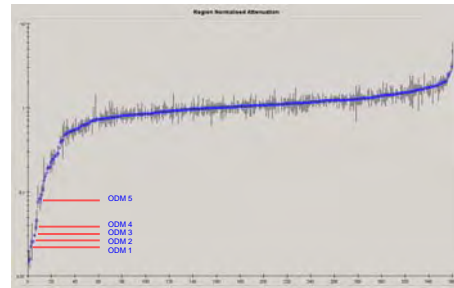
The attenuation index (i.e. relative fluorescence per gene in input pool vs output pool)



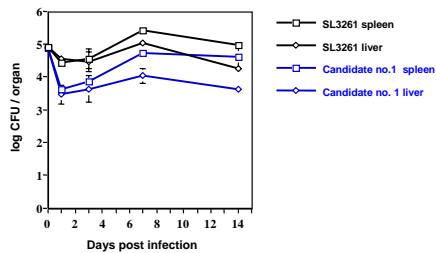
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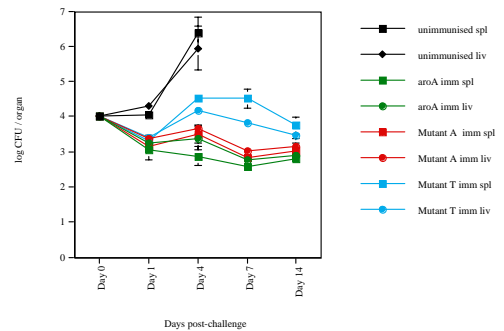
Vaccine candidates 1-5 ?



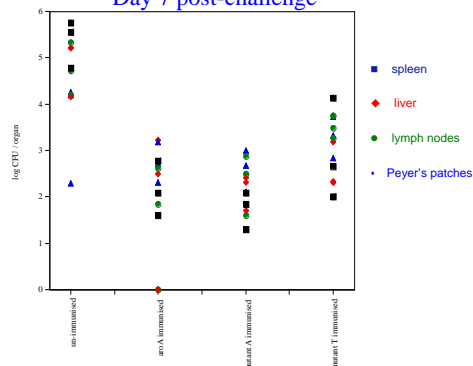
Vaccine candidate



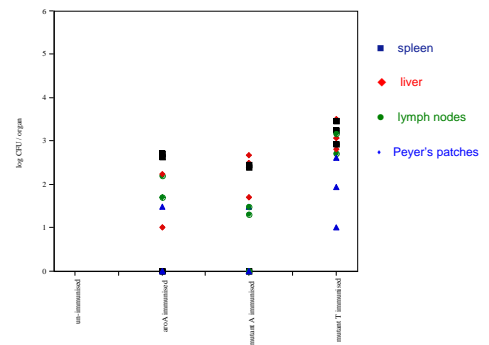
i.v. immunisation with 10⁵ mutant bacteria followed by i.v. challenge with 10⁴ wild type *S. Typhimurium*



i.v. immunisation with 10^5 mutant bacteria followed
by oral challenge with 10^8 wild type *S. Typhimurium*
Day 7 post-challenge



i.v. immunisation with 10^5 mutant bacteria followed
by oral challenge with 10^8 wild type *S. Typhimurium*
Day 14 post-challenge



Practical outcomes?

- Potential antigens for sub-unit vaccines
- Potential attenuating mutations for live vaccines
- Easier investigation of the functional genomics of infection in livestock (current BBSRC grant with IAH Compton to look at a single *Salmonella* mutant library through cattle, pigs and chickens: host specificity)

Acknowledgements

- University of Cambridge
 - Andrew Grant, Chris Coward, Claire Woodall
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- IAH Compton
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 - Arrow Therapeutics Ltd
 - BBSRC
 - BBSRC/DTI Applied Genomics LINK
 - MAFF/Defra