Genomic analysis of Salmonella enterica has lead to a better understanding of human specific pathogens

The study of microbial pathogens in the last 10 years has been characterised by a great increase in genomic analysis techniques. The human specific pathogen *Salmonella enterica* serovar Typhi is the poorly understood disease causing agent of typhoid fever. Newer high throughput technologies have accelerated our understanding of its mechanisms of survival, specificity and virulence. This increase in knowledge will lead to more efficient treatments and a decrease in global prevalence.

Salmonella enterica are a major class of motile gram negative animal pathogens which cause a diverse array of host-specific and generalist diseases. They can be classified according to the flagellar H antigen to give over 2400 serovars. Typhoid fever, a severe systemic infection, is caused by *S.enterica* serovar Typhi (*S.Typhi*) (Raffatellu *et al.*, 2008). It is transmitted through ingestion of faecal particles due to unsanitary conditions, thus it is endemic in large parts of the developing world. Although improved sanitation in recent years has led to a slight decrease in cases, typhoid fever is still a major problem and antibiotic resistance is increasing. Study of *S.Typhi* has been problematic due to its specificity to humans; no animal model can be produced which will replicate the symptoms shown in humans. For this reason, *S.enterica* serovar Typhimurium (*S.Typhimurium*) has been studied in mice with a mutation in the Nramp1 protein which replicates a systemic infection (Raffatellu *et al.*, 2008). *S.Typhimurium* is a generalist pathogen which normally causes gastroenteritis when contaminated food is consumed. Although *S.Typhimurium* and *S.Typhi* are very closely related, significant differences in their genome mean that any conclusions drawn from animal studies of *S.Typhimurium* have to be interpreted very carefully when applied to the *S.Typhi* infection of humans.

Genomic analyses now give us a better understanding of the methods of pathogenicity and reasons for host specificity seen in *S.Typhi*. In addition to this, comparative analysis allows a clearer

interpretation of results derived from animal studies using *S.Typhimurium* in relation to human *S.Typhi* infections.

Genome sequences are now available for two *S.Typhi* strains; Ty2 was isolated in the early 1900s and has been widely used in studies around the world, whilst CT18 is a multidrug resistant strain first isolated in Vietnam in 1992 (Deng *et al.*, 2003, Parkhill *et al.*, 2001). Other isolates have also been analysed, but as yet the sequences have not been published.

The emergence of new sequencing techniques will likely speed up the process of identifying variation among *S.Typhi* isolates. The majority of sequencing thus far has relied on the sanger technique of random DNA restriction and amplification of fragments with incorporation of one of four alternately coloured fluorescent dideoxynucleotides (ddNTPs). The fragments are then separated using a capillary based agarose gel, and a laser to read which colour base has been incorporated. This is converted into base sequence using computer software (Shendure and Ji, 2008). Newer techniques, such as 454 sequencing and Solexa sequencing, use cyclic array technology. After random fragmentation, each individual section is amplified to produce an individual colony of fragments. These fragments are hybridised to microarray chips, and cyclic incorporation of fluorescent ddNTPs occurs like in the Sanger method. The stationary chip allows nucleotide additions to be read whilst sequencing continues thus using fewer reagents and decreasing time and cost (Shendure and Ji, 2008).

BLAST sequence analysis is a well established tool which allows a user to input a DNA sequence of a region of interest, and find the homologous region in related species, noting changes which have occurred (Selander *et al.*, 1990). Using this technique, it was hypothesised that the *S.Typhi* group were a relatively recent branch in the Salmonella family (Boyd *et al.*, 1996). In the 1990s, with an increase in data being produced from sequencing, BLAST sequence alignment alone couldn't cope with the ability to compare multiple strains of the species. Multilocus sequencing techniques (MLST) were developed instead, where core genomic elements of different isolates can be compared and phylogenetic similarity calculated (Maiden *et al.*, 1998). The classic method of classifying bacteria

uses the similarity of phenotypic characteristics such as surface expressed antigens to determine phylogeny; however this could cause erroneous grouping of species whose phenotype has converged, rather than those with shared evolutionary history. Based on BLAST and MLST analysis, phylogenetic groups can be determined on actual sequence analysis; *S.Typhi* strains have a high degree of sequence conservation, indicating they evolved from the same precursor; lack of significant divergence from this precursor also indicates that *S.Typhi* diverged from other *S.enterica* species as recently as 30,000 years ago (Kidgell *et al.*, 2002, Selander *et al.*, 1990, Boyd *et al.*, 1996). Both these points raise interesting questions as to how *S.Typhi* has adapted to specifically infect humans in such a short period of time. It is here that comparative analysis of the *S.Typhi* and *S.Typhimurium* genomes becomes useful. *S.Typhimurium* shares the majority of its genome backbone with Escherichia coli (McClelland *et al.*, 2001); however significant changes have taken place in *S.Typhi*. Gene degradation is an accumulation of mutations within RNA and protein coding genes which prevent translation. The resulting sequences are termed pseudogenes. Although pseudogenes are common in the human genome, they are relatively rare in free living bacteria due to the necessity to minimise wasted metabolic energy (Frank *et al.*, 2002). Analysis of the *S.Typhi* CT18 genome indicated the presence of over 200 pseudogenes, which is equivelant to 5% of its genome (Parkhill *et al.*, 2001). The corresponding genes in *S.Typhimurium* are mainly intact, where only 0.69% of the genome is made up of pseudogenes (McClelland *et al.*, 2001). Gene inactivation is a relatively rapid process which may go some way to explaining how *S.Typhi* has become a human-specific pathogen in only 30,000 years. Analysis of the type of genes which have become inactivated may provide clues to determine what selective pressures have been present.

The salmonella group has previously been assessed to have a wide variety of Fimbrial genes, which can be classified into 12 groups (Parkhill *et al.*, 2001, McClelland *et al.*, 2001). In *S.Typhi*, seven of those groups have been inactivated, and this could be either a cause or effect of a restriction in host-range (Townsend *et al.*, 2001). A large number of pseudogenes have also been found in salmonella pathogenicity islands (SPIs). SPIs are regions of the genome where virulence traits tend to be

concentrated; SPI-1 and SPI-2 are found in a wide range of salmonella species and code for type three secretion systems (T3SS). They contain a broad array of genes which have been shown to be essential for invasion and survival (Mills *et al.*, 1995). A loss of these general virulence traits which are coded for by SPIs could explain decrease the host-range observed in *S.Typhi* (Townsend *et al.*, 2001).

Another factor which could have allowed for such a fast adaptation to a human host is the ability for large sections of the *S.Typhi* genome to relocate. A recent study showed that ribosomal RNA operons are encoded by seven genes. Random fragmentation can occur, with fragments being re-integrated in random order. Inversions of gene sequences were identified in 50% of the samples, leading to transcriptional silence of one or more of the ribosomal RNA genes (Kothapalli *et al.*, 2003). This level of genomic plasticity could increase the organisms' ability to adapt quickly to changes in environment (Liu and Sanderson, 1996), however a similar system has been identified in a number of other enteric bacteria thus it does not explain the rapid differences between *S.Typhi* and *S.Typhimurium*.

Foreign sections of the genome can be identified by searching for sequences which vary in certain characteristics from the majority. Codon usage within a genome is non random; it is likely that a species will selectively use one codon for an amino acid more than other homologous codons (Salim and Cavalcanti, 2008). The reason for this is due to efficiency – each separate codon requires the corresponding tRNA to allow formation of a protein. The larger number of tRNA molecules needed causes overall increase in biosynthesis which uses more energy (Charlesworth and Eyre-Walker, 2006). Pinpointing areas where codon usage varies indicates the incorporation of foreign DNA into the genome, for example a prophage sequence or an incorporated plasmid. As this section of DNA will have not co-evolved with the rest of the genome, transcriptional efficiency will not yet have been maximized (Vernikos and Parkhill, 2006). A large number of these regions have been identified in Salmonella species, and many are labelled SPIs due to the large number of virulence genes coded within them. SPI-7 is a 134Kb segment found solely in *S.Typhi* (Seth-Smith, 2008). Two loci, *viaA* and

viaB, are of particular interest due to their control of the Vi capsule (Kolyva *et al.*, 1992). The *ViaB* locus has been associated with increased survival within phagocytes, a step required for the systemic dissemination of bacteria without raising an immune response. This allows *S.Typhi* to cause a persistant infection such as Typhoid fever. *S.Typhimurium*, lacking the *ViaB* locus, causes epithelial cells to release interleukin 8 into the intenstinal mucosa due to activation of Toll like receptor (TLR) 4 and TLR5 (Wilson *et al.*, 2008). The resulting inflammation leads to an influx of neutrophils causing a short lived infection. *TviA* is a sequence within *ViaB* which represses flagellin secretion; when the *ViaB* locus was inserted into *S.Typhimurium* a decrease in invasiveness into epithelial cells was observed, but the associated inflammation was also reduced. The Vi capsule can therefore be identified as a specific adaption to allow persistence in a human host (Seth-Smith, 2008, Wilson *et al.*, 2008).

An interesting complication is the sigma factor RpoS; this is a genomic sequence which codes for a regulator of many extra-genomic virulence factors, including Vi synthesis (Santander *et al.*, 2007). This shows that although virulence may be obtained through rapidly acquired plasmids and phage sequences there are also intrinsic genomic regulators which control their expression; this can be crucial in timing the release of virulence factors to have maximum benefit to the bacteria (Fang *et al.*, 1992).

The genomic analyses which lead to the understanding of the Vi-loci have allowed the production of an efficacious vaccine. By injecting the Vi antigen, the human neutrophil mediated immune response can recognise this and prevent systemic dissemination of *S.Typhi* to create a short lived infection more similar to that caused by *S.Typhimurium*, and more easily treated with a short course of antibiotics (Lee *et al.*, 2007). Vaccines, however, do not provide a cost-effective or long term solution to the problem of typhoid fever.

A technique now commonly used in microbiology is micro-array. A transcriptome can be produced to show the functional output of a genome under certain environmental conditions. The limitation

of this technique is that small changes in environment could produce large changes in gene expression, thus experimental design is crucial. Isolating bacteria during a human infection is difficult to the small number of cells, and RNA transcripts from these cells are easily degraded, thus currently only *in vitro* studies have been performed. *S.Typhi* was infected into plated human macrophages; a known target of *S.Typhi* in the body (Faucher *et al.*, 2006). Transcriptomes were obtained from infected macrophages 2, 8 and 24 hours post infection; 117 genes were shown to upregulated at all post infection time points, and of these 19 were not found in *S.Typhimurium*. Although the Vi loci has been identified as a crucial part of *S.Typhi* infection of humans, it wasn't upregulated following infection into macrophages. This suggests that although they are necessary for the development of a latent infection, they are not required for initial survival within the human host. The *pmr* operon was a region shown to be upregulated during macrophage infection (Faucher *et al.*, 2006); it codes for a two component regulator system *PhoPQ* which controls the expression of hundreds of genes within salmonella species (Gunn *et al.*, 2000) including those essential for lipopolysaccaride modifications. It can be hypothesised that changes in expressed lipopolysaccharides during infection would highly regulate the host range of the bacteria. A *PhoPQ* knock out strain had decreased survival in plated human macrophages, showing it is required for long term survival in a human host (Baker *et al.*, 1999). Transcriptome analyses like this are useful as they give a global view of events occurring within the cell at a particular time; further *in vivo* studies would be useful so that data could be accumulated to show global changes in gene expression under particular conditions in both *S.Typhi* and *S.Typhimurium*, this data could then show subtle differences in expression between the species and be used to direct further study to particular areas of the genome where differences are concentrated.

Using similar tools to that of chromosomal analysis, such as next generation sequencing and microarray assay, characterization of non-genomic elements found in *S.enterica* has contributed to the understanding of how the rapid process of genetic variation has occurred. Most structural and

functional components are coded in the bacterial genome. Plasmids generally contribute virulence factors and because these are independent of those required for survival, horizontal gene transfer is a fast and efficacious method of creating diversity.

Antibiotic resistance is a microbial adaptation in response to human defence mechanisms. It can be viewed as an integral part host-pathogen co-evolution, thus is constantly changing (Dionisio *et al.*, 2005). In endemic diseases such as typhoid fever it is essential to understand the mechanisms by which antibiotic resistance is occurring, and often this requires widespread genomic analysis (Daigle, 2008). Antibiotic resistance conferred via plasmids in *S.Typhi* was first reported in the 1960s. Most of these plasmids were in the incompatability groups IncA and IncC, however these failed to spread widely, perhaps due to a high cost associated with carrying the plasmid (Phan and Wain, 2008). IncH were initially rarer, but conferred long term resistance against chloramphenicol, the main drug used to treat typhoid fever (Taylor and Grant, 1977). IncHI1 is now the main source of multidrug resistant *S.Typhi* (MDRST) (Phan and Wain, 2008). The sequences analysis of three IncHI1 plasmids has allowed us to look at how human drug usage has directed the evolution of *S.Typhi*; R27 (180Kb), pHCM1 (218Kb) and pAKU1 (212Kb) share a common backbone sequence, and all have the characteristic IncHI1 replication mechanism. Three initiation proteins, RepHI1A, RepHI1B, and RepHI1A-like, are present in each plasmid. The first two proteins are sufficient to replicate the entire plasmid, but the third protein appears to prevent the replication of any other plasmid type, perhaps in a competitive manner. This may explain the monopoly these plasmids have in *S.Typhi*. The reduced cost associated with this plasmid type is partially due to the large number of resistance genes present. pHCM1 confers resistance against chloramphenicol, tetracycline, streptomycin and sulphonamides, and all genes can be transcribed in one operon, thus reducing the cost of transcription (Phan and Wain, 2008). It is thought that the continued presence of the selective pressure, in this case chloramphenicol, causes evolution of the plasmid within the bacteria causing a lower fitness burden. In laboratory conditions cells which have co-evolved with the plasmid and antibiotic achieve a higher fitness than those without, even once the antibiotic has been removed

(Dionisio *et al.*, 2005). The fact that the plasmid is stably maintained in cells once the pressure has been removed indicates that integration has occurred between the plasmid and the bacterial genome, and now it is indispensable (Wain *et al.*, 2003). IncHI1 plasmids do not encode resistance against fluoroquinolones, the current drugs of choice against enteric fever, however IncHI1 are still widely found in outbreaks around the world. It will be interesting to see how carriage and composition of IncHI1 changes in response to these new drugs.

In summary, we can conclude that a number of factors have contributed to the rapid adaptation of *S.Typhi* to the human host. Genomic analysis techniques have allowed an in depth study of the molecular mechanisms which have been altered between *S.Typhi* and *S.Typhimurium*. It is likely that gene degradation of unnecessary protein family members is the largest contributing factor to the change in host range. The Vi antigen system provides *S.Typhi* with the ability to cause a latent infection, and IncHI1 plasmids confer widespread resistance against antibiotics. The greater understanding of the differences between *S.Typhi* and *S.Typhimurium* should lead to more effective experiment design in the future, and hopefully result in an efficacious therapeutic control against the global problem of typhoid fever.

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