6 Final Discussion

Conjugative plasmids are important vehicles for the horizontal gene transfer events that shape the evolution of prokaryotic organisms. In clinical settings, the rapid dissemination of antibiotic resistant determinants is mediated by the combination of integrona as assembly platforms, transposons as intracellular transfer vehicles, and conjugative plasmids as vehicles for intercellular transfer. Through one conjugative event, a large array of antibiotic resistant genes can be transferred across bacterial species or genera. Bacteria such as *S.* Typhi have gained resistance to most antibiotics available, even third generation cephalosporins, potentially leaving azithromycin the only choice for treatment in many areas.

The majority of studies on outbreaks of drug resistant *S.* Typhi were performed in developing world and occasionally in developed world when a traveller brought back the infection. Techniques such as PFGE and plasmid profiling are widely used and are great tools to study local outbreaks. However it becomes difficult to tell whether a strain brought back by a traveller is the same outbreak strain from a remote village in, say, India. Attempts have been made to address the capability to compare strain identities across laboratories, either by standardising current methods (PulseNet or EnterNet) or develop new methods (MLST, SNP typing). For the purposes of studying global epidemiology and evolution, even standardised PFGE shows its drawbacks as it is more likely to overestimate the distance between isolates. Multilocus sequence typing, developed as a tool to study global epidemiology and evolution, undoubtedly serves us better than PFGE for these purposes. SNP typing also has an overwhelm advantage over PFGE: the readiness to compare strain identities. However, it will take time before laboratories in developing countries can routinely use newer method like MLST, mostly

because of the limited availability of sequencing facilities, which are becoming faster and cheaper.

Unfortunately, the studies of plasmids in clinical settings are lagging behind. RFLP for plasmids, probably the equivalent of PFGE for bacterial chromosome with similar drawbacks, are not even routinely performed, and the current MLST methods predominantly focus only on chromosomal sequences. The only tool for the identification and classification of plasmids are replicon typing, or more recently, the PCR-based version of replicon typing. This method groups plasmids into incompatibility groups without actually performing the classical incompatibility tests. The PCR replicon typing method has recently gained popularity due to its ease of use and interpretation. The Inc groups are however too broad for the close investigation of plasmid transfer, as well as the transfer of the antibiotic resistant determinants they carry. While *S.* Typhi are endemic in developing countries such as India, Pakistan etc., only a few studies perform RFLP and even less use PCR replicon typing. Our understanding of plasmid transmission and drug resistance transmission in *S.* Typhi is therefore very limited.

Evidence has suggested the co-evolution of IncHI1 plasmids in *S.* Typhi backgrounds since the introduction of chemotherapy for the treatment of typhoid fever. Plasmids of IncHI1 are the main carriers of antibiotic resistant genes in *S.* Typhi. Given the situation, I investigated the genetic factors that contribute to stabilise the plasmid-background co-existence. Second, realising the lack of tools for the investigation of plasmids in general and IncHI1 plasmids in particular, I proposed to develop a new typing tool for IncHI1 plasmids. And lastly, I demonstrated how the simultaneous typing of both the plasmid and the background strain is useful to study the transmission of plasmids within a bacterial population.

The co-evolution of a conjugative plasmid and its bacterial host over several hundred generations potentially results in a fitter strain suggesting the plasmid and the bacterial chromosome have found a way to better co-exist. Plasmids IncHI1 have been isolated from *S.* Typhi for nearly 40 years. Studies on the global regulator H-NS have suggested a mechanism for an IncHI1 plasmid to minimise its adverse effect on a *S.* Typhi chromosome. I present in chapter 3 a high-throughput method to screen for novel genes responsible for the stable inheritance of plasmid IncHI1 in *S.* Typhi.

Transposon-directed insertion site sequencing (TraDIS) is a method that combines the commonly used Tn*5* transposon to generate random mutation libraries with a next generation sequencing technique from Illumina (formerly Solexa) to produce a high-resolution screening. I applied this method to an attenuated *S.* Typhi strain harbouring an IncHI1 plasmid and used serial passages as the selective pressure to screen for candidate genes that might contribute to plasmid stability. I identified several candidate genes on the chromosome and on the plasmids. Whilst most of the chromosomal candidates are more likely to have a role in helping the bacteria survive in the presence of chloramphenicol, the antibiotic used to select for plasmids, seven plasmid-encoded hypothetical genes, along with known genes for plasmid stability such as *parA*, *parB, sfh* and *hok,* were identified as candidates for plasmid stability. A Δ*sfh* plasmid was generated and the *S.* Typhi strain harbouring this mutant plasmid shows no growth defects compared to the same *S.* Typhi background with a wild-type plasmid. This result suggests that the effect of plasmid stability genes can be subtle. To follow up with this work, a set of carefully designed experiments, including long-term experiments and plasmid competition assays, is needed to further access the impact of plasmid stability genes. The seven candidate genes described here should be systematically knocked out and subjected to such experiments for evaluating their role in plasmid stability.

TraDIS is a novel method to take advantage of the next generation sequencing technology to simultaneously identify with high confident and high accuracy the insertion sites of more than one million mutants. This will take all the previously applications using random mutant libraries to a new level of power by using more mutants in one experiment. The applications range for this kind of library-based sequencing is huge, including, but not limiting to, defining the essential gene sets, defining genes required for the survival in different niches, defining genes required for adhesion and invasion of host cells or genes required for infection and transmission in animal models. Potential drawbacks to applying this method to other organisms is the availability of a suitable transposon with a high enough transfer rate for the generation of a large library of a million mutants. Care should also be taken in the interpretation of the results as genes that are not directly involved in the measured phenotype might be co-selected. Our example of such a situation is the co-selection of chloramphenicol resistant genes on the chromosome in the assay intended to select for plasmid stability genes because of the presence of chloramphenicol in the media.

In providing tools to investigate the population structure and evolution of IncHI1 plasmids, I applied the concept of MLST to plasmid. Six conserved genes were chosen for the IncHI1 plasmid typing scheme called IncHI1 plasmid multilocus sequencing typing (PMLST). The scheme involves amplifying and sequencing of six loci on the plasmid for their identification and classification. A collection of IncHI1 plasmid investigated using PMLST revealed two main groups of IncHI1 plasmids: group 1 consists of older plasmids isolated before 1993 and group 2 of more recent plasmids isolated after 1993. The disappearance of group 1 plasmids after 1993 suggests a replacement of group 1 by group 2 plasmids in current *S.* Typhi population. The mechanisms for this replacement remains unclear at this point but we are able to think

of two possibilities: the spread of group two plasmids to different *S.* Typhi backgrounds, driving the older plasmids out of the bacterial population – a direct plasmid competition; or the spread of a particular strain carrying a group 2 plasmids – bacterial competition, plasmid most likely to contribute to the success of this competition.

Our concept of using MLST for plasmids was also independently used by García-Fernández *et al.* to IncI1 plasmids (Garcia-Fernandez *et al.* 2008). This on one hand shows the usefulness of this method to study the evolution of plasmids but on the other hand highlights a disadvantage: the need for a separate gene set for each plasmid Inc group due to the phylogenetic distance and high level of sequence variations. Recently, Garcillan-Barcia describes the use of relaxase gene to study the evolution and classification of all conjugative and mobile plasmids (Garcillan-Barcia, Francia & de la Cruz 2009). This might provide a generic tool for the identification and typing of all plasmids but their level of discrimination between closely related plasmid requires further investigation.

In order to answer the question about the mechanism by which group 2 replaces group 1 plasmids, I made use of a newly developed SNP typing scheme to genotype *S.* Typhi chromosome and IncHI1 plasmid simultaneously. A previous study by Holt *et al.* describing the whole genome sequences of 19 *S.* Typhi isolates selected from different branches of *S.* Typhi phylogenetic tree has identified a set of SNPs (on both chromosome and plasmid) from these sequences. The SNPs were then used to develop a SNP typing method based on the Illumina GoldenGate platform. The application of this typing method on a collection of 473 *S.* Typhi isolates revealed striking data on the movement of IncHI1 plasmid within *S.* Typhi populations and highlights the emergence of a background-plasmid combination, H58/PST6, that is spreading globally. By

simultaneously typing of plasmid and chromosome, we are able to identify a strong association of a group 2 plasmid type, PST6, with the background H58. This suggests PST6 displays background specificity and that the spread of this plasmid type is the result of the successful spread of a single background strain type. This plasmid-background specificity has never been reported previously. It would be interesting to better understand the distinct contribution of the plasmid and chromosome to the success of this H58/PST6 strain. We were able to a culture based experiment to compete an older plasmid from group 1 (PST1) and PST6 in an identical Ty2-derived background (H10). The results were in apparent contrast with what is observed in nature: PST1 outcompetes PST6 in H10 background. This might suggest that PST6 requires an H58 background to show its beneficial effect, although many other factors could play a role in this artificial experiment. Further investigation is needed to fully understand the relationship of PST6 and the specific background H58.

Our SNP typing method has proved that simultaneous tracing of plasmid and background strains is very useful to provide insights to the spread of plasmids and their accompanied resistant genes in a pathogen bacterial population. This data will provide information for the management of drug resistance in the future.

Unlike MLST which is a variation discovery method where newly emerged strains will be added to the database, SNP typing uses a defined set of known SNPs for the typing of isolates which means that newly emerged strains carrying new SNP loci will be missed by SNP typing. This drawback can be addressed by periodical addition of new SNP loci to the current typing set. This requires the continuing use of SNP discovery methods such as MLST and re-sequencing.

In conclusion, there is still much to learn about the interactions between a plasmid and its host's chromosome in terms of their stable co-existence. Many interactions are likely

to be subtle and need specially designed experiments to investigate. Although the role of plasmids in the dissemination of antibiotic resistance has been established and widely known, detailed reports on plasmids from clinical isolates are rare due to technological limitations. This study presents the details of PMLST, a typing and classification scheme for IncHI1 plasmids, and a SNP typing scheme to simultaneously trace plasmids and their host genetic backgrounds. Future work will assess the utility of these approaches to demonstrate their value to study the evolution and transmission of plasmids in clinical isolates within the context of their bacterial host population.

Publications arising from this Thesis

Gemma C. Langridge*, Minh-Duy Phan*, Daniel J. Turner*, Timothy T. Perkins, Leopold Parts, Jana Haase, Ian Charles, Duncan J. Maskell, Sarah E. Peters, Gordon Dougan, John Wain, Julian Parkhill and A. Keith Turner. 2009, "Simultaneous assay of every Salmonella Typhi gene using one million transposon mutants", *Genome Research*, vol. 19, no. 12, pp. 2308-2316.

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Phan, M. D., Kidgell, C., Nair, S., Holt, K. E., Turner, A. K., Hinds, J., Butcher, P., Cooke, F. J., Thomson, N. R., Titball, R., Bhutta, Z. A., Hasan, R., Dougan, G. and Wain, J. 2009, "Variation in Salmonella enterica serovar typhi IncHI1 plasmids during the global spread of resistant typhoid fever", *Antimicrob. Agents Chemother*, vol. 53, no. 2, pp. 716-727.

Phan, M.D., Wain, J. 2008, "IncHI1 plasmids, a dynamic link between resistance and Pathogenicity", *J. Infect. Developing Countries*, vol. 2, no. 4, pp. 272-278.

Holt, K. E., Thomson, N. R., Wain, J., Phan, M. D., Nair, S., Hasan, R., Bhutta, Z. A., Quail, M. A., Norbertczak, H., Walker, D., Dougan, G. and Parkhill, J. 2007, "Multidrug- resistant Salmonella enterica serovar paratyphi A harbors IncHI1 plasmids similar to those found in serovar typhi", *J. Bacteriol*, vol. 189, no. 11, pp. 4257-4264.