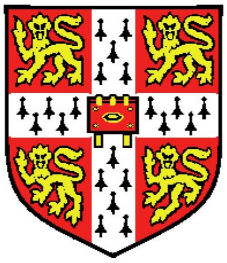
High-throughput Experimental and Computational Studies of Bacterial Evolution



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Arrakis teaches the attitude of the knife – chopping off what's incomplete and saying:
"Now it's complete because it's ended here."

Collected Sayings of Muad'dib

Declaration

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The work presented in this dissertation was carried out at the Wellcome Trust Sanger Institute between October 2009 and August 2013. This dissertation is the result of my own work and includes nothing which is the outcome of work done in collaboration except where specifically indicated in the text. This dissertation does not exceed the limit of 60,000 words as specified by the Faculty of Biology Degree Committee. This dissertation has been typeset in 12pt Computer Modern font using LaTeX according to the specifications set by the Board of Graduate Studies and the Faculty of Biology Degree Committee. No part of this dissertation or anything substantially similar has been or is being submitted for any other qualification at any other university.

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It's been a gas.

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Abstract

The work in this thesis is concerned with the study of bacterial adaptation on short and long timescales. In the first section, consisting of three chapters, I describe a recently developed high-throughput technology for probing gene function, transposon-insertion sequencing, and its application to the study of functional differences between two important human pathogens, *Salmonella enterica* subspecies *enterica* serovars Typhi and Typhimurium. In a first study, I use transposon-insertion sequencing to probe differences in gene requirements during growth on rich laboratory media, revealing differences in serovar requirements for genes involved in iron-utilization and cell-surface structure biogenesis, as well as in requirements for non-coding RNA. In a second study I more directly probe the genomic features responsible for differences in serovar pathogenicity by analyzing transposon-insertion sequencing data produced following a two hour infection of human macrophage, revealing large differences in the selective pressures felt by these two closely related serovars in the same environment.

The second section, consisting of two chapters, uses statistical models of sequence variation, i.e. covariance models, to examine the evolution of intrinsic termination across the bacterial kingdom. A first collaborative study provides background and motivation in the form of a method for identifying Rho-independent terminators using covariance models built from deep alignments of experimentally-verified terminators from *Escherichia coli* and *Bacillus subtilis*. In the course of the development of this method I discovered a novel putative intrinsic terminator in *Mycobacterium tuberculosis*. In the final chapter, I extend this approach to *de novo* discovery of intrinsic termination motifs across the bacterial phylogeny. I present evidence for lineage-specific variations in canonical Rho-independent terminator composition, as well as discover seven non-canonical putative termination motifs. Using a collection of publicly available RNA-seq datasets, I provide evidence for the function of some of these elements as *bona fide* transcriptional attenuators.

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List of Symbols

Roman Symbols

A, C, G, T, U	Adenine, Cytosine, Guanine, Thymine, Uracil
Fe(II)	Ferrous iron
Fe(III)	Ferric iron

Greek Symbols

λ	Phage lambda
σ^E	σ^{24}, extracytoplasmic stress sigma factor
σ^S	σ^{38}, starvation/stationary phase sigma factor

Amino Acids

Ala, A	Alanine
Arg, R	Arginine
Asn, N	Asparagine
Asp, D	Aspartic acid (Aspartate)
Cys, C	Cysteine
Gln, Q	Glutamine
Glu, E	Glutamic acid (Glutamate)

Gly, G	Glycine
His, H	Histidine
Ile, I	Isoleucine
Leu, L	Leucine
Lys, K	Lysine
Met, M	Methionine
Phe, F	Phenylalanine
Pro, P	Proline
Ser, S	Serine
Thr, T	Threonine
Trp, W	Tryptophan
Tyr, Y	Tyrosine
Val, V	Valine

Acronyms and Abbreviations

BALB	Bagg albino (mouse)
BLAST	Basic local alignment search tool
bp	Base pair
CCAL	Creative Commons attribution license
CCD	Charge-coupled device
CDP	Cytidine diphosphate glucose
ChIP-seq	Chromatin immunoprecipitation sequencing
cI	Clear 1 (λ repressor protein)

CM	Covariance model
CPM	Counts per million (reads)
CYK	Cocke-Younger-Kasami (algorithm)
ddNTP	dideoxynucletoide
DeADMAn	Designer microarrays for defined mutant analysis
DNA	Deoxyribonucleic acid
dNTP	deoxynucleotide
DSB	Disulfide bond
DUS	DNA uptake sequence
E-value	Expect value
ECA	Enterobacterial common antigen
EHEC	Enterohemorrhagic *Escherichia coli*
EM	Expectation-maximization
FASTA	Fast alignment
FDR	False discovery rate
FMN	Flavin mononucleotide
FPR	False positive rate
GEBA	Genomic encyclopedia of bacteria and archea
GLM	Generalized linear model
GO	Gene ontology
HIRAN	HIP116, Rad5p N-terminal
HITS	High-throughput insertion tracking by deep sequencing

HMM	Hidden Markov model
iid	Independent identically distributed (random variable)
INSeq	Insertion sequencing
kb	Kilobase
KEGG	Kyoto encyclopedia of genes and genomes
LEE	Locus of enterocyte effacement
LLR	$\log_2$-likelihood ratios
logFC	Log_2 fold-change
LPS	Lipopolysaccharide
MATT	Microarray tracking of transposon mutants
Mb	Megabase
MCC	Matthews correlation coefficient
MFE	Minimum free energy
MIS	Most informative sequence
ncRNA	non-coding RNA
NOGD	Nonorthologous gene displacement
OMP	Outer membrane protein
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PCA	Principal component analysis
PCR	Polymerase chain reaction

PMA	Phorbol myristate acetate
PPV	Positive predictive value
RIT	Rho-independent terminator
RNA	Ribonucleic acid
RNA-seq	RNA sequencing
RNAP	RNA polymerase
RNase	Ribonuclease
RPMI	Rosewell Park Memorial Institute (cell culture medium)
RSS	Reciprocal similarity score
SAGE	Serial analysis of gene expression
SCV	*Salmonella* containing vacuole
SPI	*Salmonella* pathogenicity island
SPV	*Salmonella* plasmid virulence (genes)
sRNA	Bacterial small RNA
SRP	Signal recognition particle
STM	Signature-tagged mutagenesis
T3SS	Type III secretion system
TAM	terminus associated motif
TMM	Trimmed mean of M-values
tmRNA	Transfer-messenger RNA
Tn-seq	Transposon mutagenesis and sequencing
TNF-α	Tumor necrosis factor α

TraDIS	Transposon directed insertion sequencing
TraSH	Transposon site hybridization
TRIT	Tuberculosis Rho-independent terminator
tRNA	Transfer RNA

Introduction

Bacteria possess a remarkable ability to adapt. This ability has allowed bacteria to colonize almost every environment on Earth, from deep sea hydrothermal vents (Jørgensen et al., 1992) to cryogenic brine lakes (Murray et al., 2012) to animal hosts (Finlay et al., 1997). Indeed, the ability of bacteria to establish symbiotic relationships with host cells was a critical step in the origin of so-called "higher" eukaryotic life (Sagan, 1967). While the origins of some bacterial adaptations are buried in the deep time of over 1.5 billion years of evolution (Doolittle et al., 1996), such as the differing bauplans observed across phyla, others are far more recent, such as the emergence of *Yersinia pestis* as a human pathogen around 20,000 years ago (Achtman et al., 1999) or the contemporary development of specialized invasive lineages of non-typhoidal *Salmonella* in immunocompromised individuals in sub-Saharan Africa (Feasey et al., 2012; Okoro et al., 2012). Many factors likely contribute to this continuous adaptation, including large population sizes, short generation times, wide-spread homologous recombination between related strains, and a capacity for horizontal gene transfer. These factors, particularly homologous recombination and horizontal gene transfer, make the definition of species in bacteria contentious (Achtman et al., 2008; Doolittle et al., 2009), and have led to some questioning the viability of a bacterial species concept altogether. For the present I will leave these matters to those better informed than myself, and work within the established, though flawed, taxonomy.

The work in this thesis is concerned with the study of bacterial evolution and adaptation on two very different time scales. In the first section, consisting of chapters 1, 2, and 3, I describe a recently emerged high-throughput technology for probing gene function, transposon-insertion sequencing (Barquist et al., 2013a), and its application to the study of functional differences in two important human pathogens, *Salmonella enterica* subspecies *enterica* serovars Typhi and Typhimurium. These two serovars

diverged only approximately 50,000 years ago (Kidgell et al., 2002), yet have developed very different host ranges and cause very different diseases, with *S.* Typhi causing a life-threatening systemic disease exclusively in humans, and *S.* Typhi causing primarily a mild gastrointestinal disease in a wide range of hosts. Chapter 2 uses transposon-insertion sequencing to probe differences in gene requirements during growth on rich laboratory media, revealing differences in requirements for genes involved in iron-utilization and cell-surface structure biogenesis, as well as in requirements for non-coding RNA (Barquist et al., 2013b). In chapter 3 I more directly probe the genomic features responsible for differences in serovar pathogenicity by analyzing transposon-insertion sequencing data produced following a two hour infection of human macrophage, revealing large differences in the selective pressures felt by these two closely related strains in the same environment.

The second section, chapters 4 and 5, uses statistical models of sequence variation, i.e. covariance models, to examine the evolution of intrinsic termination across the bacterial kingdom. Chapter 4 provides background and motivation in the form of a method for identifying Rho-independent terminators using covariance models built from deep alignments of experimentally-verified terminators from *Escherichia coli* and *Bacillus subtilis* (Gardner et al., 2011). In the course of the development of this method I discovered a novel putative intrinsic terminator in *Mycobacterium tuberculosis*. In chapter 5, I extend this approach to *de novo* discovery of intrinsic termination motifs across the bacterial phylogeny. I present evidence for lineage-specific variations in canonical Rho-independent terminator composition, as well as discover seven non-canonical putative termination motifs. Using a collection of publicly available RNA-seq datasets, I provide evidence for the function of these elements as *bona fide* transcriptional attenuators.

Chapter 1

Querying bacterial genomes with transposon-insertion sequencing

This chapter is an expansion of the previously published article "Approaches to querying bacterial genomes using transposon-insertion sequencing" (Barquist et al., 2013a). Amy K. Cain and Christine J. Boinett (Pathogen Genomics, Wellcome Trust Sanger Institute) contributed to the research of the original article. All final language is my own.

1.1 Introduction

The study of gene essentiality has its roots in evolutionary theory, systems biology, and comparative genomics, and has been instrumental in the development of the emerging discipline of synthetic biology. Koonin summarizes the major scientific motivation behind this line of research succinctly: "When reverse-engineering a complex machine, one basic goal is to draw up a list of essential parts" (Koonin, 2003). The earliest attempt at constructing such a minimal gene set involved a comparison between the first two complete genomes sequenced: *Mycoplasma genitalium* and *Haemophilus influenzae* (Mushegian et al., 1996). Both of these organisms are pathogens with highly reduced genomes; however, they are derived from distant branches of the bacterial phylogeny being Gram-positive and -negative, respectively. Orthology prediction based on sequence similarity identified 240 genes shared between the two organisms. However, a number of essential pathways were found to be incomplete in this set due to non-orthologous

gene displacement (NOGD), and a true minimal gene set was estimated to contain 256 genes. NOGD apparently occurs when an unrelated but functionally analogous gene is introduced in a lineage, and subsequently the ancestral gene is lost. The sequencing of complete genomes has shown that this phenomena is surprisingly wide-spread, and only ∼60 genes appear to be universally conserved (Koonin, 2003). Rather obviously in hindsight, it appears that gene essentiality is highly dependent on the evolutionary and systems context in which the gene occurs - our essential parts list depends on the machine we wish to build.

Large-scale experimental studies seem to confirm this. A range of approaches have been taken to experimentally determining the 'essential' genes of a diverse array of organisms. These include plasmid-insertion mutagenesis in *Bacillus subtilis* (Kobayashi et al., 2003), antisense-mediated gene inactivation in *Haemophilus influenzae* (Akerley et al., 2002), transposon mutagenesis in *Pseudomonas aerguinosa* (Jacobs et al., 2003), and insertion-duplication mutagenesis in *Salmonella enterica* (Knuth et al., 2004). However, the "gold standard" for the determination of gene essentiality is repeated failure to generate targeted single gene deletions. Comprehensive single gene deletion libraries libraries have been created for the γ-proteobacteria *E. coli* and *Acinetobacter baylyi* (Baba et al., 2006; Berardinis et al., 2008) where λ-red mediated recombineering has simplified the generation of defined deletions (Datsenko et al., 2000), though the process is still extremely labor-intensive. Typical estimates for essential gene sets determined by these various techniques range from less than 300 to 600 genes, depending on the organism. This variability is likely dependent on a variety of factors, including false positives and negatives due to experimental techniques, the growth conditions of the experiment, intrinsic properties of the cell being manipulated, and accidents of evolution. Now that it has become feasible to synthesize a viable bacterial chromosome (Gibson et al., 2010), a deeper understanding of the factors affecting gene requirements in diverse conditions is the next hurdle on the road to engineering truly synthetic life.

A common approach to identifying genomic regions required for survival under a particular set of conditions is to screen large pools of mutants simultaneously. This can be done with defined mutants (Baba et al., 2006; Hobbs et al., 2010), but this is both labor-intensive and requires accurate genomic annotation, which can be particularly difficult to define for non-coding regions. An alternative to defined libraries is the construction and analysis of random transposon-insertion libraries. The original application of this method

used DNA hybridization to track uniquely tagged transposon-insertions in *Salmonella enterica* serovar Typhimurium over the course of BALB/c mouse infection (Hensel et al., 1995). DNA hybridization was eventually superseded by methods that used microarray detection of the genomic DNA flanking insertion sites, variously known as TraSH, MATT, and DeADMAn (reviewed in Mazurkiewicz et al., 2006). However, these methods suffered from many of the problems microarrays generally suffer from: difficulty detecting low-abundance transcripts, mis-hybridization, probe saturation, and difficulty identifying insertion sites precisely.

The application of high-throughput sequencing to the challenge of determining insertion location and prevalence solves many of these problems. Interestingly, the first application of transposon-insertion sequencing, developed by Hutchison et al. (1999), actually predates the development of microarray-based methods. However, this was applied to libraries of only approximately 1000 transposon mutants in highly reduced *Mycoplasma* genomes, and the difficulty of sequencing at the time prevented wide spread adoption or high resolution. Modern high-throughput sequencing technology allows the methods discussed in this chapter to routinely monitor as many as one million mutants simultaneously in virtually any genetically tractable microorganism.

Table 1.1: Summary of transposon-insertion sequencing studies to date.

Study: Hutchison et al. (1999) **Organism(s):** *M. genitalium*, *M. pneumoniae*	**Application:** Gene requirements **Total mutants:** 1291 **Insertion density:** 1/850 bp **Transposon:** Tn*4001* **Name coined:** GTM
Study: Goodman et al. (2009) **Organism(s):** *B. thetaiotaomicron*	**Application:** Gene requirements for colonization of a murine model of the human gut **Total mutants:** 2 X 35,000 **Insertion density:** 1/182 bp **Transposon:** Mariner **Name coined:** INSeq
Study: Gawronski et al. (2009) **Organism(s):** *H. influenzae*	**Application:** Prolonged survival in the murine lung **Total mutants:** 75,000 **Insertion density:** 1/32 bp **Transposon:** Mariner **Name coined:** HITS
Study: Opijnen et al. (2009) **Organism(s):** *S. pneumoniae*	**Application:** Transcriptional regulation and carbohydrate transport **Total mutants:** 6 x 25,000 **Insertion density:** 1/91 bp **Transposon:** Mariner **Name coined:** Tn-seq
Study: Langridge et al. (2009) **Organism(s):** *S.* Typhi	**Application:** Gene requirements, bile tolerance **Total mutants:** 1.1 million **Insertion density:** 1/13 bp

	Transposon: Tn*5* **Name coined:** TraDIS
Study: Gallagher et al. (2011) **Organism(s):** *P. aeruginosa*	**Application:** Tobramycin resistance **Total mutants:** 100,000 **Insertion density:** 1/65 bp **Transposon:** Mariner **Name coined:** Tn-seq (circle method)
Study: Eckert et al. (2011) **Organism(s):** *E. coli*	**Application:** Colonization of bovine intestinal tract; retrospective re-evaluation of a STM study **Total mutants:** 19 x 95 **Insertion density:** 1/65 bp **Transposon:** Tn*5*
Study: Christen et al. (2011) **Organism(s):** *C. crescentus*	**Application:** Genomic requirements **Total mutants:** 800,000 **Insertion density:** 1/8 bp **Transposon:** Tn*5*
Study: Griffin et al. (2011) **Organism(s):** *M. tuberculosis*	**Application:** Gene requirements and cholesterol utilization **Total mutants:** 2 X 100,000 **Insertion density:** 1/120 bp **Transposon:** Mariner
Study: Khatiwara et al. (2012) **Organism(s):** *S.* Typhimurium	**Application:** Bile, starvation, and heat tolerance **Total mutants:** 16,000 **Insertion density:** 1/610 bp **Transposon:** Tn*5*
Study: Mann et al. (2012) **Organism(s):** *S. pnuemoniae*	**Application:** Determining roles of sRNAs in pathogenesis **Total mutants:** 9,000-24,000 **Insertion density:** Varying **Transposon:** Mariner

Study: Opijnen et al. (2012) **Organism(s):** *S. pnuemoniae*	**Application:** Stress response and metabolism *in vitro* and murine *in vivo* colonization **Total mutants:** 4,000 - 30,000 **Insertion density:** Varying **Transposon:** Mariner
Study: Brutinel et al. (2012) **Organism(s):** *S. oneidensis*	**Application:** Gene requirements and metabolism **Total mutants:** 50,000 **Insertion density:** 1/191 bp **Transposon:** Mariner
Study: Zhang et al. (2012) **Organism(s):** *M. tuberculosis*	**Application:** Genomic requirements **Total mutants:** 2 x 100,000 **Insertion density:** 1/120 bp **Transposon:** Mariner
Study: Klein et al. (2012) **Organism(s):** *P. gingivalis*	**Application:** Gene requirements **Total mutants:** N/A **Insertion density:** 1/43 bp **Transposon:** Mariner
Study: Pickard et al. (2013) **Organism(s):** *S.* Typhi	**Application:** Requirements for survival of bacteriophage infection **Total mutants:** 1.1 million **Insertion density:** 1/13 bp **Transposon:** Tn*5*
Study: Barquist et al. (2013b) **Organism(s):** *S.* Typhi, *S.* Typhimurium	**Application:** Comparison of genomic requirements between two *Salmonella* serovars **Total mutants:** 1.1 million, 930,000 **Insertion density:** 1/13 bp, 1/9 bp **Transposon:** Tn*5*

1.2 Protocols

Several methods were developed concurrently for high-throughput sequencing of transposon-insertion sites: TraDIS (Langridge et al., 2009), INSeq (Goodman et al., 2009), HITS (Gawronski et al., 2009), and Tn-seq (Opijnen et al., 2009) followed by Tn-seq Circle (Gallagher et al., 2011) and refinements to the INSeq protocol (Goodman et al., 2011). All of these protocols follow the same basic workflow with minor variations (see Figure 1.1; Table 1.1): transposon mutagenesis and construction of pools of single insertion mutants; enrichment of transposon-insertion junctions; and finally, in some protocols a purification step either precedes or follows PCR enrichment before sequencing.

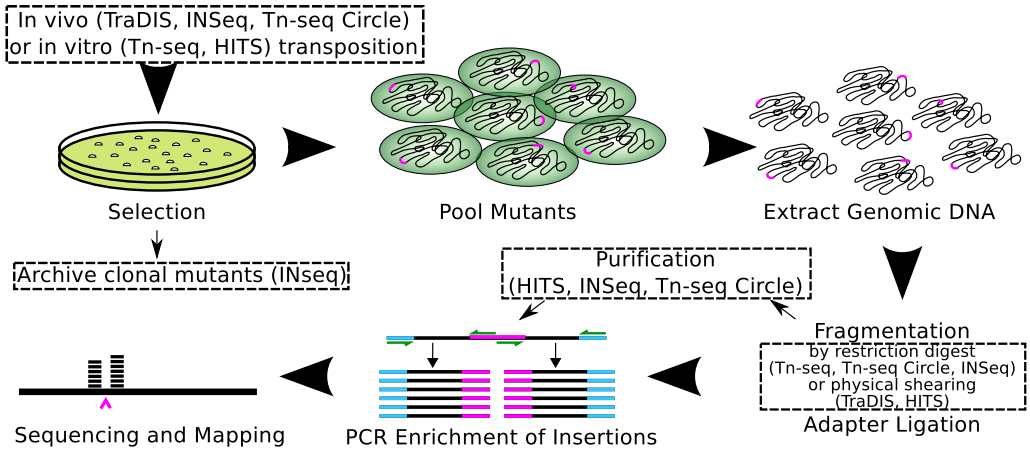


Figure 1.1: Transposon-insertion sequencing protocols. An illustration of the workflow typical of transposon-insertion sequencing protocols. Transposons are represented by pink lines, sequencing adaptors by blue, genomic DNA by black, and PCR primers by green. Mutants are generated through either *in vivo* or *in vitro* transposition and subsequent selection for antibiotic resistance. These mutants are pooled, and optionally competed in test conditions, then genomic DNA is extracted and fragmented by restriction digest or physical shearing. Sequencing adaptors are ligated, some protocols then perform a step to purify fragments containing transposon insertions, and PCR with transposon- and adapter-specific primers is used to specifically enrich for transposon-containing fragments. The fragments are then sequenced and mapped back to a reference genome to uniquely identify insertion sites with nucleotide-resolution. Dashed boxes indicate steps which differ between protocols.

1.2.1 Transposon mutagenesis

Most studies have used either Tn*5* or Mariner transposon derivatives. Tn*5* originated as a bacterial transposon which has been adapted for laboratory use. Large-scale studies have shown that Tn*5*, while not showing any strong preference for regional GC-content, does have a weak preference for a particular insertion motif (Shevchenko et al., 2002; Adey et al., 2010; Green et al., 2012). Transposon-insertion sequencing studies performed with Tn*5* transposons in *S.* enterica serovars have reported a slight bias towards AT-rich sequence regions (Langridge et al., 2009; Barquist et al., 2013b). However, this preference does not appear to be a major obstacle to analysis given the extremely high insertion densities obtained with this transposon (Langridge et al., 2009; Christen et al., 2011; Barquist et al., 2013b) (see Table 1.1). Additionally, Tn*5* has been shown to be active in a wide range of bacterial species, though the number of transformants obtained can vary significantly depending on the transformation efficiency of the host.

Mariner *Himar1* transposons on the other hand originate from eukaryotic hosts and have an absolute requirement for TA bases at their integration site (Lampe et al., 1998; Rubin et al., 1999), with no other known bias besides a possible preference for bent DNA (Lampe et al., 1998). This can be a disadvantage in that it limits the number of potential insertion sites, particularly in GC-rich sequence. However, this specificity can also be used in the prediction of gene essentiality in near-saturated libraries: as every potential integration site is known and the probability of integration at any particular site can be assumed to be roughly equal, it is straight-forward to calculate the probability that any particular region lacks insertions by chance. *Himar1* transposition can also be conducted *in vitro* in the absence of any host factors (Lampe et al., 1996), and inserted transposons can then be transferred to the genomes of naturally transformable bacteria through homologous recombination (Johnsborg et al., 2007). This can be advantageous when working with naturally transformable bacteria with poor electroporation efficiency (Gawronski et al., 2009; Opijnen et al., 2009). It is worth noting that Tn*5* is also capable of transposition *in vitro* (Goryshin et al., 1998), and could potentially be used to increase insertion density and hence the resolution of the assay, particularly in GC-rich genomic regions.

1.2.2 Pool construction

Once mutants have been constructed, they are plated on an appropriate selective media for the transposon chosen, and colonies are counted, picked, and pooled. A disadvantage of this is that the mutants must be recreated for follow up or validation studies. Goodman et al. introduced a clever way around this in the INSeq protocol: by individually archiving mutants, then sequencing combinatorial mutant pools it is possible to uniquely characterize $2n$ insertion mutants by sequencing only n pools (Goodman et al., 2009). Each mutant is labelled with a unique binary string that indicates which pools it has been added to. These binary strings can then be reconstructed for each insertion observed in these pools by recording their presence or absence in sequencing data, providing a unique pattern relating insertions to archived mutants. The authors control false identifications due to errors in sequencing by requiring that each binary label have a minimum edit distance to every other label, allowing for a robust association of labels with insertions despite sometimes noisy sequencing data. As a proof of concept, the authors were able to identify over 7,000 *Bacteroides thetaiotaomicron* mutants from only 24 sequenced pools. This effectively uses methods for the generation of random transposon pools to rapidly generate defined mutant arrays, though it is heavily dependent on liquid-handling robotics.

1.2.3 Enrichment of transposon-insertion junctions

Once pools have been constructed they are grown in either selective or permissive conditions, depending on the experiment, and then genomic DNA is extracted. Fragmentation proceeds either through restriction digestion in the case of transposons modified to contain appropriate sites (Goodman et al., 2009; Opijnen et al., 2009; Gallagher et al., 2011) or via physical shearing (Langridge et al., 2009; Gawronski et al., 2009), then sequencing adapters are ligated to the resulting fragments. PCR is performed on these fragments using a transposon-specific primer and a sequencing adapter-specific primer to enrich for fragments spanning the transposon-genomic DNA junction.

Some protocols purify fragments containing transposon insertions using biotinylated primers (Gallagher et al., 2011; Goodman et al., 2011) or PAGE (Goodman et al., 2009) before and/or after PCR enrichment. The purification step from the Tn-seq Circle protocol is particularly unusual in that restriction digested fragments containing

transposon sequence are circularized before being treated with an exonuclease that digests all fragments without transposon insertions, theoretically completely eliminating background (Gallagher et al., 2011). Given the success of protocols that do not include a purification step and the lack of systematic comparisons, it is currently unclear whether including one provides any major advantages.

1.2.4 Sequencing

The protocol steps described so far are broadly similar to those used in microarray-based studies of transposon mutant pools. The major advancement that has driven transposon-insertion sequencing has been the recent development of second generation DNA sequencing technologies. For 30 years, DNA sequencing was dominated by dideoxynucleotide, or Sanger, sequencing, first described by Sanger et al. (1977). Sanger sequencing requires a clonal population of template DNA molecules, to which a primer and a full complement of four deoxynucleotides (dNTPs) and a single species of dideoxynucleotide (ddNTP) are added. DNA polymerase is then used to perform rounds of DNA extension, with ddNTPs stochastically terminating the reaction, before the resulting fragments are denatured and separated with gel electrophoresis. By running four such reactions with each species of ddNTP, the sequence of the template molecule can be determined by reading off bands on the gel. A number of advancements progressively improved the throughput and decreased the cost of Sanger sequencing, including the substitution of capillary electrophoresis for gel electrophoresis and the use of fluorescently labelled ddNTP (fluorescent dye-terminator sequencing) enabling sequencing in a single reaction. However, even with these advances the throughput of Sanger sequencing remained in the range of kilobases of sequence per hour, and costs remained high due to requirements for template cloning and inherent limitations in the technology (Morozova et al., 2008).

The development of second generation sequencing technologies in the early-mid 2000's broke these barriers to the adoption of sequencing as a routine experimental technique. These technologies include Roche 454 pyrosequencing, Illumina/Solexa reversible terminator sequencing, and ABI SOLiD parallel sequencing by ligation. While in principle any of these technologies could be applicable to transposon-insertion sequencing, all studies to date have used Solexa sequencing. Solexa sequencing is similar in principle to Sanger

sequencing, with two major innovations: the ability to generate arrayed clonal clusters of template molecules on a glass flow cell (described by Fedurco et al. (2006)) allowing for hundreds of thousands of simultaneous sequencing reactions, and the adoption of reversible dye terminator chemistry (described by Bentley et al. (2008)) which allows for fluorescently labelled terminators to be rapidly stripped of their fluorophore, their termination reversed, and extension continued. By monitoring successive rounds of these hundreds of thousands of parallel sequencing reactions with a CCD camera, the sequence of a large population of template molecules can be determined quickly and simultaneously, leading to current throughputs of megabases to gigabases of sequence per hour. As each resulting read corresponds to a single template molecule, this technology is ideally suited to monitoring populations of transposon mutants, providing an accurate digital count of insertion prevalence.

1.3 Reproducibility, accuracy, and concordance with previous methods

A number of studies have looked at the reproducibility of transposon-insertion sequencing. Multiple studies using different protocol variations have repeatedly shown extremely high reproducibility in the number of insertions per gene (correlations of 90%) in replicates of the same library grown and sequenced independently (Goodman et al., 2009; Opijnen et al., 2009; Gallagher et al., 2011), and good reproducibility (correlations between 70-90%) in independently constructed unsaturated libraries (Opijnen et al., 2009; Opijnen et al., 2012). Opijnen et al. (2012) compared traditional 1 X 1 competition experiments between wild-type and mutant *Streptococcus pneumoniae* to results obtained by transposon-insertion sequencing and showed that there was no significant difference in results over a range of tested conditions. The accuracy of transposon-insertion sequencing in determining library composition has also been assessed. Zhang et al. (2012) constructed a library of identified transposon-insertion mutants in known relative quantities, and then were able to recover the relative mutant prevalence with transposon-insertion sequencing. Additionally, by estimating the number of PCR templates prior to enrichment, this study showed that there is a high correlation between enrichment input and sequencing output.

Two studies have evaluated concordance between results obtained with transposon-

insertion sequencing and microarray monitoring of transposon insertions in order to demonstrate the enhanced accuracy and dynamic range of sequencing over previous methods. In the first, 19 libraries of 95 enterohemorrhagic *Escherichia coli* (EHEC) transposon mutants that had previously been screened in cattle using signature-tagged mutagenesis (STM) were pooled and re-evaluated using the TraDIS protocol (Eckert et al., 2011). The original STM study had identified 13 insertions in 11 genes attenuating intestinal colonization in a type III secretion system located in the locus of enterocyte effacement (LEE) (Dziva et al., 2004). By applying sequencing to the same samples, an additional 41 mutations in the LEE were identified, spanning a total of 21 genes. Additional loci outside the LEE which have been previously implicated in intestinal colonization but had not been detected by STM were also reported by TraDIS.

The second study re-evaluated genes required for optimal growth determined by TraSH in *Mycobacterium tuberculosis* (Sassetti et al., 2003; Griffin et al., 2011). The greater dynamic range of sequencing as compared to microarrays allowed easier discrimination between insertions that were truly unviable and those that were only significantly underrepresented. The authors estimate that genes called as required by sequencing in their study are at least 100-fold underrepresented in the pool. In comparison, the threshold in the previous microarray experiment reported genes that had log probe ratios at least 5-fold lower than average between transposon-flanking DNA hybridization and whole genomic DNA hybridization. Additionally, the nucleotide-resolution of insertion sequencing allowed the authors to identify genes which had required regions, likely corresponding to required protein domains (Zhang et al., 2012), but which tolerated insertions in other regions. Altogether the authors increase the set of genes predicted to be required for growth in laboratory conditions in *M. tuberculosis* by more than 25% (from 614 to 774).

1.4 Identifying gene requirements

The earliest application of transposon-insertion sequencing, and indeed the earliest genome-wide experimental study of gene essentiality, was to determine the minimal set of genes necessary for the survival of *Mycoplasma* (Hutchison et al., 1999). This essential genome is of great interest in synthetic and systems biology where it is seen as a foundation for engineering cell metabolism as described previously, and also in infection

biology and medicine where it is seen as a promising target for therapies. However, it is important to remember that essentiality is always relative to growth conditions: a biosynthetic gene that is non-essential in a growth medium supplying a particular nutrient may become essential in a medium that lacks it. Traditionally, gene essentiality has been determined in clonal populations (Baba et al., 2006; Jacobs et al., 2003; Glass et al., 2006); since the high-throughput transposon sequencing protocols described here necessarily contain a short period of competitive growth before DNA extraction, many of these studies prefer to refer to the required genome for the particular conditions under evaluation.

Because of this short period of competitive growth, and because many otherwise required genes tolerate insertions in their terminus (Goodman et al., 2009; Griffin et al., 2011; Zomer et al., 2012) or outside essential domains (Zhang et al., 2012) the determination of required genomic regions is not completely straight-forward and a number of approaches have been taken to counter this. These include only calling genes completely lacking insertions as required (Opijnen et al., 2009), or determining a cut-off based on the empirical or theoretical distribution of gene-wise insertion densities (Langridge et al., 2009; Barquist et al., 2013b; Griffin et al., 2011; Zomer et al., 2012). Additionally, windowed methods have been developed which can be used to identify essential regions in the absence of gene annotation (Zhang et al., 2012; DeJesus et al., 2013), and have had success in identifying required protein domains, promoter regions, and non-coding RNAs (ncRNAs). The organisms that have been evaluated for gene requirements under standard laboratory conditions are summarized in Table 1.1. In agreement with previous studies (Baba et al., 2006; Jacobs et al., 2003), many required genes identified by transposon-insertion sequencing are involved in fundamental biological processes such as cell division, DNA replication, transcription and translation (Langridge et al., 2009; Goodman et al., 2009; Barquist et al., 2013b; Griffin et al., 2011), and many of these requirements appear to be conserved between genera and classes (Barquist et al., 2013b; Christen et al., 2011).

However, a recent study defining required gene sets in *Salmonella* serovars (described in detail in the next chapter) has found that phage repressors, necessary for maintaining the lysogenic state of the prophage, are also required (Barquist et al., 2013b), even though mobile genetic elements such as phage are usually considered part of the accessory genome. This study also highlights the need for temperance when interpreting the

results of high-throughput assays of gene requirements. For example, many genes in *Salmonella* Pathogenicity Island 2 (SPI-2) did not exhibit transposon-insertions, despite clear evidence from directed knockouts showing that these genes are non-essential for viability or growth. Under laboratory conditions, SPI-2 is silenced by the nucleoid-forming protein H-NS (Lucchini et al., 2006; Navarre et al., 2006), which acts by oligermerizing along silenced regions of DNA blocking RNA polymerase access. A previous study has shown that transposon insertion cold spots can be caused by competition between high-density proteins and transposases for DNA (Manna et al., 2007). This suggests that H-NS may be restricting transposase access to DNA, though this has not previously been observed in transposon-insertion sequencing data, and will require additional work to confirm.

1.5 Determining conditional gene requirements

One of the most valuable applications of the transposon-insertion sequencing method is the ability to identify genes important in a condition of interest, by comparing differences in the numbers of sequencing reads from input (control) mutant pools to output (test) pools that have been subject to passaging in a certain growth condition. Insertion counts are compared from cells in the input pool and those after passage, thereby identifying genes that either enhance or detract from survival and/or growth in the given condition, defined by decreased or increased insertion frequency, respectively. A further application of this method involves comparing insertions between biologically linked conditions, such as cellular stresses or different stages of a murine infection, to gain insight into complex systems (Opijnen et al., 2012).

So far, transposon-insertion sequencing has been used to investigate a number of interesting biologically relevant conditions: bile tolerance in *S.* Typhi (Langridge et al., 2009) and *S.* Typhimurium (Khatiwara et al., 2012), bacteriophage infection of *S.* Typhi (Pickard et al., 2013), antibiotic resistance in *P. aeruginosa* (Gallagher et al., 2011), cholesterol utilization in *M. tuberculosis* (Griffin et al., 2011) and survival in number of stress and nutrient conditions in *S. pneumoniae* (Opijnen et al., 2012). Transposon-insertion sequencing of populations passed through murine models have been used to assess genes required for *H. influenzae* infection (Gawronski et al., 2009). A further extension of the method examined double mutant libraries, that is transposon mutant

libraries generated in a defined deletion background, to tease apart complex networks of regulatory genes (Opijnen et al., 2009).

Two studies in particular illustrate the power of using transposon-insertion sequencing to identify conditionally required genes. In the first, Goodman et al. (2009) set out to determine the genes necessary for the establishment of the commensal *B. thetaiotaomicron* in a murine model. First, the growth requirements of transposon mutant populations in the cecum of germ-free mice was assessed, and genes required for growth in monoassociation with the host were found to be enriched in functions such as energy production and amino acid metabolism. By further comparing monoassociated transposon mutant libraries with those grown in the presence of three defined communities of human gut-associated bacteria, the authors identified a locus up-regulated by low levels of vitamin B12 that is only required in the absence of other bacteria capable of synthesizing B12. This showed that the gene requirements of any particular bacterium in the gut are at least partially dependent on the metabolic capabilities of the entire community and emphasizes the importance of testing *in vivo* conditions to complement *in vitro* study.

The second study, conducted by Opijnen et al. (2012), aimed to map the genetic networks involved in a range of cellular stress responses in *S. pneumoniae*. Seventeen *in vitro* conditions were tested, including: pH, nutrient limitation, temperature, antibiotic, heavy metal, and hydrogen peroxide stress. Approximately 6% of disrupted genes resulted in increased fitness in some condition, suggesting that some genes are maintained despite being detrimental to the organism under particular conditions. These would be interesting candidates for further functional and evolutionary study, as the maintenance of these genes is presumably highly dependent on the conditions the bacteria faces, and may have implications for our understanding of e.g. gene loss in the process of bacterial host adaptation (Toft et al., 2010). Two additional *in vivo* experiments were performed in a murine model, where cells were recovered from the lung and nasopharynx. Combining this data, over 1,800 genotype-phenotype genetic interactions were identified. These interactions were mapped and pathways identified. Between the two *in vivo* niches, certain stress responses pathways were markedly different. For example, temperature stress produced a distinct response in the lung, compared to the nasopharyanx, which is perhaps to be expected as temperature varies greatly between these two sites. By further examining sub-pathways required in the two different niches and comparing them to *in vitro* requirements, the authors were able to draw conclusions regarding the condition

S. pneumoniae faces when establishing an infection. This comprehensive mapping of genotype-phenotype relationships will serve as an important atlas for further studies of the factors affecting *S. pneumoniae* carriage and virulence.

1.6 Monitoring ncRNA contributions to fitness

To date, four studies (including one described in detail in the next chapter) have used transposon-insertion sequencing to examine the contribution of non-coding RNAs (ncRNAs) and other non-coding regions to organismal fitness (see Table 1.1). Two of these examined requirements for non-coding regions in the relatively under-explored bacterial species *Caulobacter crescentus* (Christen et al., 2011) and *M. tuberculosis* (Zhang et al., 2012). Both utilized analytical techniques that allowed for the identification of putative required regions in the absence of genome annotation. Twenty-seven small RNAs (sRNAs) had previously been detected in *C. crescentus* (Landt et al., 2008); 6 were found to be depleted in transposon insertions indicating an important role in basic cellular processes. Additionally, the well-characterized ncRNAs tmRNA and RNase P, as well as 29 non-redundant tRNAs were found to be required. An additional 90 unannotated non-disruptable regions were identified throughout the genome, implying an abundance of unexplored functional non-coding sequence.

While the non-coding transcripts of *M. tuberculosis* have been explored more thoroughly than those of *C. crescentus*, most remain functionally uncharacterized, though there are hints that some of these may be involved in pathogenicity (Arnvig et al., 2012). Using a Mariner transposon-based assay and a windowed statistical analysis that accounted for the distribution of potential TA integration sites, 35 intergenic regions were identified as putatively required in the *M. tuberculosis* genome (Zhang et al., 2012). In common with the *C. crescentus* study, the RNA component of RNase P, required for the maturation of tRNAs, and tmRNA, involved in the freeing of stalled ribosomes, were identified as required (Figure 1.2 A) together with 10 non-redundant tRNAs and potential promoter regions. However, due to the lower overall insertion density and lack of TA sites in some GC-rich regions, there were some regions that could not be assayed and the resolution was limited to 250 bases.

A particularly exciting study has been conducted in *S. pneumoniae* TIGR4 combining RNA-seq with transposon-insertion sequencing (Mann et al., 2012). To identify sRNA

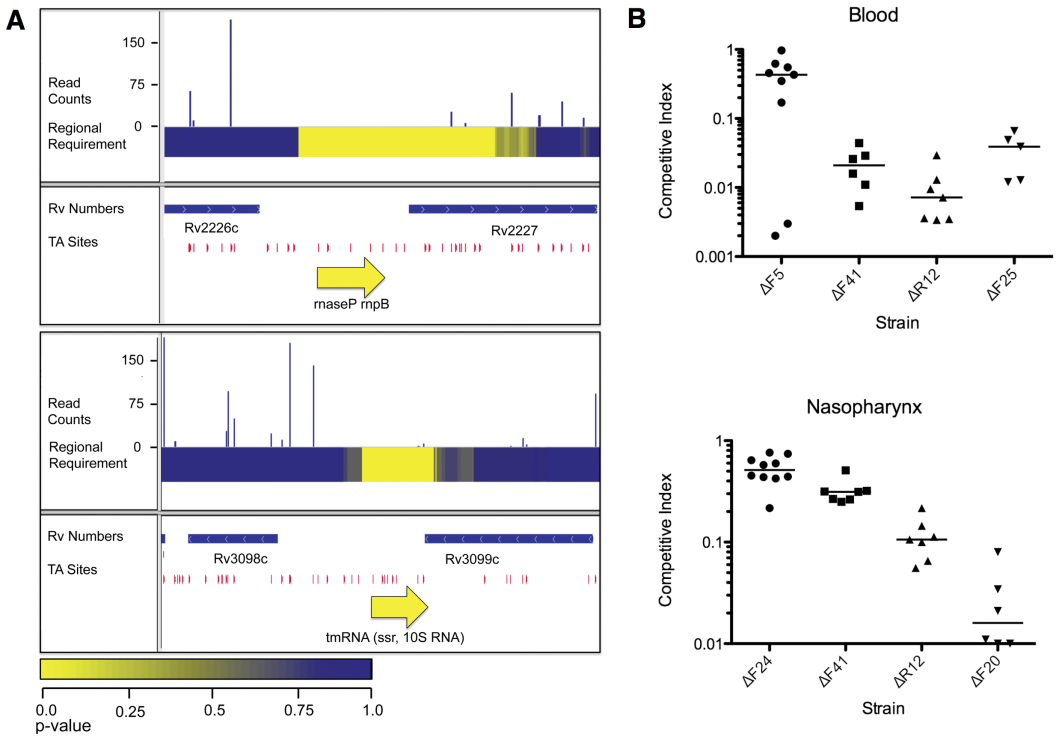


Figure 1.2: Applications of transposon-insertion sequencing to non-coding RNAs. A) Plots of genomic regions in *Mycobacterium tuberculosis* containing the required non-coding RNAs RNase P (top) and tmRNA (bottom). Tracks, from top to bottom: 1. Histogram of insertion counts, 2. Comprehensive heat-map of requirement of 500-bp windows, 3. Position of annotated genes, 4. Position of TA dinucleotide sites, 5. Position of non-coding RNA. Reproduced from Zhang et al. (2012) under a Creative Commons Attribution License (CCAL). B) 1 X 1 competition assays validate attenuating *Streptococcus pneumoniae* sRNA mutants identified by transposon-insertion sequencing. Mice were infected with defined deletions of sRNAs identified as attenuating by Tn-seq and wild type *S. pneumoniae* TIGR4 at the body site indicated and bacterial densities were compared 24 hours post-infection. These plots show the derived competitive index in blood (top) and the nasopharynx (bottom). Each point represents the result of a competition experiment between an sRNA deletion mutant and wild-type TIGR4. A competitive index of 1 indicates equivalent numbers of mutants and wild-type were recovered. Modified from Mann et al. (2012) under a CCAL.

loci the authors first sequenced size-select RNA from wild type TIGR4 and three two-component system knockouts, identifying 89 putative sRNAs, 56 of which were novel. Fifteen of these candidates, selected on the basis of high expression and low predicted folding free energy, were assayed for their ability to establish invasive disease in a murine model. Of these 8 sRNA deletions showed a significant attenuation of disease. To more broadly establish the roles of sRNAs in infecting particular organs, transposon insertion libraries were administered directly to the nasopharnyx, lungs, or blood of mice, and bacteria were harvested following disease progression. Twenty-six, 28, and 18 sRNAs were found to attenuate infection in the nasopharnyx, lung and blood respectively. These results were then validated with targeted deletions of 11 sRNAs (Figure 1.2 B). In addition to establishing the role of sRNAs in *S. pneumoniae* virulence, this study illustrated the power of combining RNA-seq and transposon-insertion sequencing to rapidly assign phenotypes to non-coding sequences.

1.7 Limitations

In this chapter, I have largely focused on the potential of transposon insertion sequencing. However, this technology does have a number of important limitations. As discussed previously, requirements for particular nucleotides at insertion sites, such as the TA required by Mariner transposons, or preference for certain sequence composition, such as the AT bias exhibited by Tn*5*, can limit the density of observed insertions in certain genomic regions. This may impact any down-stream analysis, and can potentially bias results, particularly the determination of gene requirements. Even if this bias has been accounted for, transposon-insertion screens will always over-predict gene requirements in comparison to targeted deletion libraries as discussed previously. However, this over-prediction can be controlled either through careful consideration of known insertion biases as in many Mariner-based studies, or by high insertion densities, such as those achieved in several Tn*5*-based studies (Table 1.1). Once the library has been created, only regions that have accumulated insertions in the conditions of library creation will be able to be assayed for fitness effects in further conditions. This means that regions that lead to slow growth phenotypes when disrupted in standard laboratory conditions may be difficult to assay in other conditions. Additionally, the dynamic range of fitness effects detected will depend on the complexity of the input library(s). The absence of insertions may be a

particular problem for assaying small genomic elements, such as sRNAs or short ORFs. Finally, the validation of hypotheses derived from transposon-insertion sequencing will require the construction of targeted deletions, as individual mutants cannot be recovered from pools unless specialized protocols have been followed during library construction (as in Goodman et al., 2009).

1.8 The future of transposon-insertion sequencing

Transposon-insertion sequencing is a robust and powerful technique for the rapid connection of genotype to phenotype in a wide range of bacterial species. Already, a number of studies have demonstrated the effectiveness of this method and the results have been far-reaching: enhancing our understanding of basic gene functions, establishing requirements for colonization and infection, mapping complex metabolic pathways, and exploring non-coding genomic dark matter. Due to the range of potential applications of transposon-insertion sequencing, along with the decreasing cost and growing accessibility of next-generation sequencing, I believe that this method will become increasingly common in the near future.

A number of bacterial species have already been subjected to transposon-insertion sequencing (Table 1.1). Microarray-based approaches to monitoring transposon mutant libraries have even been applied to eukaryotic systems (Ross-Macdonald et al., 1999), and similarly transposon-insertion sequencing can potentially be applied to any system where the creation of large-scale transposon mutant libraries is technologically feasible. Recently the Genomic Encyclopedia of Bacteria and Archea (GEBA) (Wu et al., 2009) has been expanding our knowledge of bacterial diversity through targeted genomic sequencing of underexplored branches of the tree of life. Applying transposon-insertion sequencing in a comparative manner across the bacterial phylogeny will provide an unprecedented view of the determinants for survival in diverse environments - the next chapter describes a study taking the first steps toward this eventual goal (Barquist et al., 2013b). While most transposon-insertion sequencing studies to date have focused on pathogenic bacteria, these techniques could also have applications in energy production, bioremediation, and synthetic biology.

The combination of transposon-insertion sequencing with other high-throughput and computational methods is already proving to be fertile ground for enhancing our under-

standing of bacterial systems. For instance, by using transposon-insertion sequencing in a collection of relatively simple conditions combined with a computational pathway analysis, Opijnen et al. (2012) were able to provide a holistic understanding of the genetic subsystems involved in a complex process such as *S. pneumoniae* pathogenesis. In the future, methods to assay phenotype in a high-throughput manner (Bochner, 2009; Nichols et al., 2011) may be combined with transposon-insertion sequencing to provide exhaustive simple genotype-phenotype associations with which to understand complex processes in a systems biology framework.

Chapter 2

A comparison of dense transposon insertion libraries in the *Salmonella* serovars Typhi and Typhimurium

This chapter is a modified version of the previously published article "A comparison of dense transposon insertion libraries in the Salmonella *serovars Typhi and Typhimurium" (Barquist et al., 2013b). This work is a result of collaboration with Gemma C. Langridge (Pathogen Genomics, Wellcome Trust Sanger Institute), who constructed the* Salmonella *Typhimurium transposon mutant library and contributed to a draft manuscript. In particular, portions of the analyses in sections 2.3.1-3 have their origins in Langridge (2010), though have been significantly elaborated on here.*

2.1 Introduction

Salmonella enterica subspecies *enterica* serovars Typhi (*S.* Typhi[1]) and Typhimurium (*S.* Typhimurium) are important, closely related, human pathogens with very different lifestyles. In this chapter, I describe a study comparing dense transposon insertion libraries created in these two serovars. The results of this study demonstrate that orthologous genes can have dramatically different effects on the fitness of recently diverged organisms

[1]Note that the complicated *Salmonella* taxonomy and nomenclature make abbreviation difficult (and at times contentious). Here I have adopted the practice of referring to individual serovars as *S.* Serovar once they have been introduced, following the advice of Brenner et al. (2000).

in rich media. These differences in fitness effects are indicative of changes in the network architecture of the cell which may partially underlie the dramatically different diseases caused by each organism and their different host ranges. Additionally, *S.* Typhimurium has served as a model organism for the discovery and functional characterization of ncRNAs. Comparing ncRNA requirements between it and a closely related serovar provides a glimpse of the functional evolution of non-coding regulatory networks.

2.1.1 The genus *Salmonella*

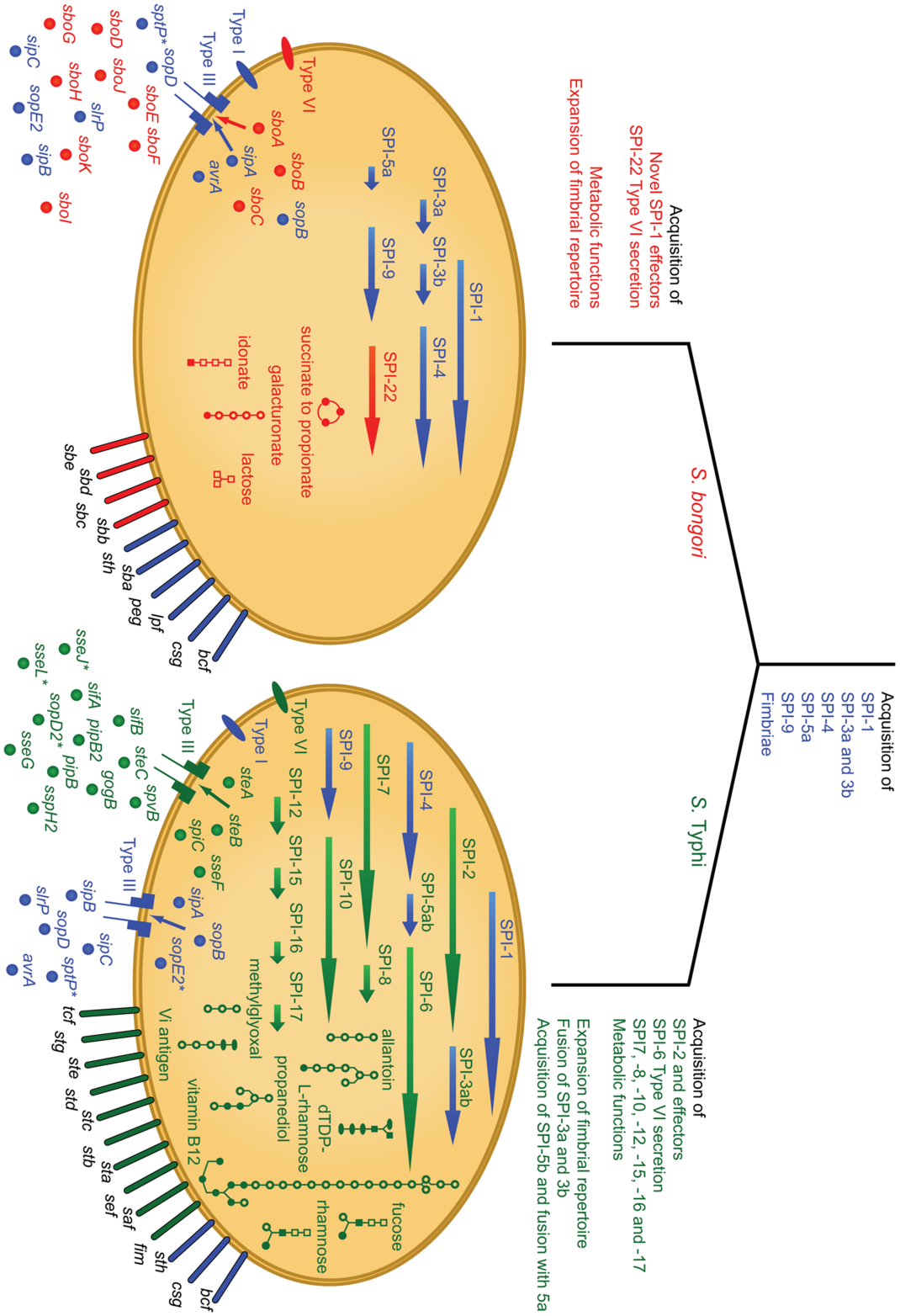
Salmonella is a Gram-negative, γ-proteobacterial genus within the order Enterobacteriales, consisting of two species: *Salmonella enterica* and *Salmonella bongori*, though a contested third species, *Salmonella subterranea*, has recently been proposed (Shelobolina et al., 2004). Based on phylogenetic analyses of 16S and conserved amino acid sequences, *Salmonella* is most closely related to the genuses *Escherichia*, *Shigella*, and *Citrobacter* (Paradis et al., 2005; Pham et al., 2007; Wu et al., 2009). Molecular clock analyses suggest that *Salmonella* and *Escherichia* shared a common ancestor between 100 and 160 million years ago (Ochman et al., 1987; Doolittle et al., 1996), though complete genetic isolation of the two genera may have taken 70 million years (Retchless et al., 2007). During the time since their divergence *Escherichia* has become established as a mammalian gut commensal, though multiple independent origins of the *Shigella* and other pathogenic phenotypes within the genus show that a disease phenotype can be developed fairly easily through the horizontal acquisition of virulence determinants and the silencing of anti-virulence loci (Kaper et al., 2004; Prosseda et al., 2012). Despite sharing the majority of their genomes with *Escherichia* and having broadly similar metabolic capabilities (AbuOun et al., 2009), the salmonellae exist primarily as pathogens, though are possibly commensal in some reptiles (Mermin et al., 2004; Bauwens et al., 2006).

The difference in dominant phenotype between *Escherichia* and *Salmonella* appears to be largely due to the acquisition of virulence determinants which opened new niches to ancestral salmonellae (see figure 2.1). Many of the virulence determinants characteristic of the salmonellae are encoded on large genomic islands with sizes between ∼6 and 140 kilobases, termed *Salmonella* Pathogenicity Islands (SPIs) (Hensel, 2004). These islands encode a diverse array of pathogenicity-related functions including secretion systems, toxins, antibiotic resistances, and lipopolysaccharide (LPS) and capsular modifications.

In particular, the acquisition of SPI-1, encoding a type 3 secretion system (T3SS), and various fimbriae by the ancestral *Salmonella* likely enabled invasion of cells in the intestinal epithelium and escape from competition with other members of the gut microbiota (Bäumler, 1997). *S. bongori* appears to have only acquired a single additional SPI since its divergence from *S. enterica* and likely retains a lifestyle more similar to the ancestral *Salmonella*, though there is evidence for additional adaptation to its niche in the reptilian gut (Fookes et al., 2011).

S. enterica meanwhile has diversified into 6 distinct subspecies: *enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae*, and *indica*. These subspecies are further divided into over 2000 serovars based on the cell-surface O, flagellar H, and capsular Vi antigens (Grimont et al., 2007). The acquisition of SPI-2, involved in survival inside macrophages and an enabling factor for systemic infection (Kuhle et al., 2004; Abrahams et al., 2006), by the ancestral *S. enterica* is thought to have been a driving force in this diversification (Bäumler, 1997). Subspecies besides *enterica* are thought to be primarily restricted to cold-blooded animals (Bäumler, 1997), though sporadic reports of zoonotic disease show these subspecies are capable of transiently colonizing the mammalian gut under certain conditions (Mermin et al., 2004; Hilbert et al., 2012). However, here I will be primarily concerned with the subspecies *enterica* and its adaptation to the mammalian, and more specifically human, host.

Figure 2.1 *(following page)*: Genomic acquisitions in the evolution of the salmonellae. Traits shared by the common ancestor are depicted in blue; those unique to *S. bongori* are shown in red and those unique to *S. enterica* subspecies *enterica* serovar Typhi in green. Arrows, *Salmonella* Pathogenicity Islands (SPIs); extended ovals, fimbriae; circles, effectors; small ovals and needle complexes, secretion systems. Metabolic pathways: lines, enzymatic reactions; open squares, carbohydrates; ovals, pyrimidines; open circles, other substrates; filled shapes, phosphorylated. Novel effectors acquired by *S. bongori* are secreted by the type III secretion system encoded on SPI-1. SPI-3a and 3b carry the same genes in both organisms but are fused into one island in *S.* Typhi. SPI-5a also carries the same genes in both organisms, but a further 3 kb (termed SPI-5b) has fused to SPI-5a in *S.* Typhi. *indicates a pseudogene. Reproduced from Fookes et al. (2011) under a Creative Commons Attribution License (CCAL).


Acquisition of
SPI-1
SPI-3a and 3b
SPI-4
SPI-5a
SPI-9
Fimbriae
S. bongori
Acquisition of
Novel SPI-1 effectors
SPI-22 Type VI secretion
Metabolic functions
Expansion of fimbrial repertoire
S. Typhi
Acquisition of
SPI-2 and effectors
SPI-6 Type VI secretion
SPI7, -8, -10, -12, -15, -16 and -17
Metabolic functions
Expansion of fimbrial repertoire
Fusion of SPI-3a and 3b
Acquisition of SPI-5b and fusion with 5a
SPI-1
SPI-3a
SPI-3b
SPI-4
SPI-5a
SPI-9
SPI-22
Type VI
Type III
Type I
sboA
sboB
sopB
sipA
sboC
avrA
sptP*
sopD
sboE
sboF
sboD
sboJ
slrP
sboG
sboH
sboK
sipC
sopE2
sipB
sboI
succinate to propionate
galacturonate
idonate
lactose
sbe
sbd
sbc
sbb
sth
sba
peg
lpf
csg
bcf
SPI-1
SPI-2
SPI-3ab
SPI-4
SPI-5ab
SPI-6
SPI-7
SPI-8
SPI-9
SPI-10
SPI-12
SPI-15
SPI-16
SPI-17
Type VI
Type III
Type I
steA
steB
spiC
sseF
sipA
sopB
sopE2*
sipB
sipC
slrP
sopD
sptP*
avrA
sifB
steC
spvB
gogB
pipB
sspH2
sseG
sopD2*
pipB2
sifA
sseJ*
sseL*
methylglyoxal
propanediol
vitamin B12
Vi antigen
allantoin
dTDP-
L-rhamnose
rhamnose
fucose
tcf
stg
ste
std
stc
stb
sfa
sef
saf
fim
sth
csg
bcf

2.1.2 Host adaptation and restriction

Bacterial adaptation to a pathogenic lifestyle is a complex process involving both the acquisition of virulence factors and gene loss through both passive decay and positive selection (Pallen et al., 2007; Prosseda et al., 2012). In the previous section I discussed how the acquisition of SPI-1 and -2, among other factors, have enabled *S. enterica* subspecies *enterica* to establish a niche in the mammalian gut. Access to this new niche has enabled serovars of subspecies *enterica* to explore a range of pathogenic modalities. The most common form of disease caused by *enterica* serovars is a self-limiting gastroenteritis, exemplified by the serovars Typhimurium and Enteriditis (Santos et al., 2009). These serovars can infect a wide range of mammals and birds, but are only capable of causing serious disease in the very young (Bäumler et al., 1998), and are generally thought to exhibit a phenotype similar to the ancestral *enterica*.

A number of subspecies *enterica* serovars have adapted to causing invasive disease in specific organisms. These include Typhi and Paratyphi in humans, Dublin in cattle, Gallinarum in chickens, Abortusovis in sheep, Choleraesuis in pigs, and Abortusequi in horses. These adaptations appear to be the result of the acquisition of host-specific virulence factors (Bäumler et al., 1998). Interestingly, those serovars associated with the most severe forms of disease appear to be most highly restricted in terms of host range. This appears to be the result of three processes: positive selection against anti-virulence loci (Pallen et al., 2007; Prosseda et al., 2012), and two more passive processes termed "use it or lose it" and "use it, but lose it anyway" by Moran (2002).

Selection against anti-virulence loci presumably occurs during host-adaptation, and generally involved the loss of loci that provoke an antigenic response or interfere with the infective process. Once a bacterium has escaped competition in the gut microbiota and gained access to a rich intracellular niche through horizontal acquisitions, the "use it or lose it" principle leads to the loss of metabolic pathways no longer required in this environment presumably due to the lifting of selective pressure for their maintenance. The "use it, but lose it anyway" principle is a consequence of the severe bottleneck imposed by adaptation to a particular host, which will often drastically reduce the effective population size of the bacterium. This can cause fixation of inactivating mutations in potentially beneficial genes simply as an accident of the adaptive process. Together these processes may eventually prevent the bacterium from living independently of its host;

particularly extreme examples are *Mycobacterium leprae* with its thousands of inactivated pseudogenes (Cole et al., 2001), *Mycoplasma* species with their highly reduced genomes (Fraser et al., 1995), and most strikingly the endosymbiont-derived mitochondria and plastid organelles (Sagan, 1967; Andersson et al., 1998). While no *Salmonella* serovars appear to have been subject to this degree of genome degradation, it is not unusual for as much as 7% of the protein-coding genes of host-restricted serovars to be inactivated (Parkhill et al., 2001; Thomson et al., 2008; Holt et al., 2009; McClelland et al., 2004).

The serovars of *S. enterica* subspecies *enterica* exhibit a spectrum of pathogenic lifestyles, from low-pathogenicity and wide host range to high-pathogenicity and narrow host range. Recent studies examining host adaptation of Typhimurium strains to immunocompromised populations (Feasey et al., 2012; Okoro et al., 2012) demonstrate that the process of host-adaptation is both on-going and highly relevant to human health. In this study, we have used transposon-insertion sequencing to examine two recently diverged (circa 50,000 years ago (Kidgell et al., 2002)) serovars at extreme ends of this pathogenicity spectrum: Typhi and Typhimurium.

2.1.3 Serovars Typhi and Typhimurium

Salmonella enterica subspecies *enterica* serovars Typhi (*S.* Typhi) and Typhimurium (*S.* Typhimurium) are important human pathogens with distinctly different lifestyles. *S.* Typhi is host-restricted to humans and causes typhoid fever. This potentially fatal systemic illness affects at least 21 million people annually, primarily in developing countries (Crump et al., 2004; Bhutta et al., 2009; Kothari et al., 2008), and is capable of colonizing the gall bladder creating asymptomatic carriers; such individuals are the primary source of this human restricted infection, exemplified by the case of "Typhoid Mary" (Soper, 1939). Mary Mallon was an Irish-American cook in New York City at the turn of the twentieth century, and an (at least initially) unwitting carrier of Typhi. A series of typhoid outbreaks were traced to her by city public health authorities. She was offered removal of her gall bladder, which she refused, and was ordered to refrain from working as a cook following release from three years of quarantine. After a number of additional outbreaks – including several deaths – were traced to Mary, who had continued working as a cook under a pseudonym, she was involuntarily quarantined on North Brother Island in the East River for 23 years until her death.

S. Typhimurium, conversely, is a generalist, causing relatively mild disease in a wide range of mammals and birds in addition to being a leading cause of foodborne gastroenteritis in human populations. Control of *S.* Typhimurium infection in livestock destined for the human food chain is of great economic importance, particularly in swine and cattle (CDC, 2009; Majowicz et al., 2010). Additionally, *S.* Typhimurium causes an invasive disease in mice, which has been used extensively as a model for pathogenicity in general and human typhoid fever specifically (Santos et al., 2001).

Despite this long history of investigation, the genomic factors that contribute to these differences in lifestyle remain unclear. Over 85% of predicted coding sequences are conserved between the two serovars in sequenced genomes of multiple strains (McClelland et al., 2001; Parkhill et al., 2001; Holt et al., 2008; Deng et al., 2003). The horizontal acquisition of both plasmids and pathogenicity islands during the evolution of the salmonellae is believed to have impacted upon their disease potential. A 100kb plasmid, encoding the *Salmonella* plasmid virulence (SPV) genes, is found in some *S.* Typhimurium strains and contributes significantly towards systemic infection in animal models (Gulig et al., 1987; Gulig et al., 1993). *S.* Typhi is known to have harbored IncHI1 plasmids conferring antibiotic resistance since the 1970s (Phan et al., 2009), and there is evidence that these strains present a higher bacterial load in the blood during human infection (Wain et al., 1998). Similar plasmids have been isolated from *S.* Typhimurium (Datta, 1962; Holt et al., 2007; Cain et al., 2012). *Salmonella* pathogenicity islands 1 and 2 are common to all *Salmonella enterica* subspecies, and are required for invasion of epithelial cells (reviewed in Darwin et al. (1999)) and survival inside macrophages respectively (Ochman et al., 1996; Shea et al., 1996; Kuhle et al., 2004; Abrahams et al., 2006). *S.* Typhi additionally incorporates SPI-7 and SPI-10, which contain the Vi surface antigen and a number of other putative virulence factors (Pickard et al., 2003; Seth-Smith, 2008; Townsend et al., 2001).

Acquisition of virulence determinants is not the sole explanation for the differing disease phenotypes displayed in humans by *S.* Typhimurium and *S.* Typhi; genome degradation is an important feature of the *S.* Typhi genome, in common with other host-restricted serovars such as *S.* Paratyphi A (humans) and *S.* Gallinarum (chickens). In each of these serovars, pseudogenes account for 4-7% of the genome (Parkhill et al., 2001; Thomson et al., 2008; Holt et al., 2009; McClelland et al., 2004). Loss of function has occurred in a number of *S.* Typhi genes that have been shown to encode intestinal

colonisation and persistence determinants in *S.* Typhimurium (Kingsley et al., 2003). Numerous sugar transport and degradation pathways have also been interrupted (Parkhill et al., 2001), but remain intact in *S.* Typhimurium, potentially underlying the restricted host niche occupied by *S.* Typhi.

In addition to its history as a model organism for pathogenicity, *S.* Typhimurium has recently served as a model organism for the elucidation of non-coding RNA (ncRNA) function (Vogel, 2009a). These include cis-acting switches, such as RNA-based temperature and magnesium ion sensors (Waldminghaus et al., 2007; Cromie et al., 2006), together with a host of predicted metabolite-sensing riboswitches. Additionally, a large number of trans-acting small RNAs (sRNAs) have been identified within the *S.* Typhimurium genome (Kröger et al., 2012), some with known roles in virulence (Hebrard et al., 2012). These sRNAs generally control a regulon of mRNA transcripts through an antisense binding mechanism mediated by the protein Hfq in response to stress. The functions of these molecules have generally been explored in either *S.* Typhimurium or *E. coli*, and it is unknown how stable these functions and regulons are over evolutionary time (Richter et al., 2012).

Transposon mutagenesis has previously been used to assess the requirement of particular genes for cellular viability. The advent of next-generation sequencing has allowed simultaneous identification of all transposon insertion sites within libraries of up to 1 million independent mutants (reviewed in Barquist et al. (2013a); see also the previous chapter), enabling us to answer the basic question of which genes are required for *in vitro* growth with extremely fine resolution. By using transposon mutant libraries of this density, which in *S.* Typhi represents on average > 80 unique insertions per gene (Langridge et al., 2009), shorter regions of the genome can be interrogated, including ncRNAs (Christen et al., 2011). In addition, once these libraries exist, they can be screened through various selective conditions to further reveal which functions are required for growth/survival.

Illumina-based transposon directed insertion-site sequencing (TraDIS (Langridge et al., 2009)) with large mutant libraries of both *S.* Typhimurium and *S.* Typhi was used to investigate whether these salmonellae require the same protein-coding and non-coding RNA (ncRNA) gene sets for competitive growth under laboratory conditions, and whether there are differences which reflect intrinsic differences in the pathogenic niches these bacteria inhabit.

2.2 Materials and Methods

Gemma Langridge created the S. *Typhimurium library described here, and performed all the laboratory experiments described here. Duy Phan and Keith Turner created the* S. *Typhi library. Duy Phan and Gemma Langridge performed the read mapping.*

2.2.1 Strains

S. Typhimurium strain SL3261 was used to generate the transposon mutant library and contains a deletion relative to the parent strain, SL1344. The 2166bp deletion ranges from 153bp within *aroA* (normally 1284bp) to the last 42bp of *cmk*, forming two pseudogenes and deleting the intervening gene SL0916 completely. For comparison, our previously generated *S.* Typhi Ty2 transposon library (Langridge et al., 2009) was used.

2.2.2 Annotation

For *S.* Typhimurium strain SL3261, I used feature annotations drawn from the SL1344 genome (EMBL-Bank accession FQ312003.1), ignoring the deleted *aroA*, *ycaL*, and *cmk* genes. I re-analyzed the *S.* Typhi Ty2 transposon library with features drawn from an updated genome annotation (EMBL-Bank accession AE014613.1.) I supplemented the EMBL-Bank annotations with non-coding RNA annotations drawn from Rfam 10.1 (Burge et al., 2013), Sittka et al. (2008), Chinni et al. (2010), Raghavan et al. (2011), and Kröger et al. (2012). Selected protein-coding gene annotations were supplemented using the HMMER webserver (Finn et al., 2011) and Pfam (Punta et al., 2012).

2.2.3 Creation of *S.* Typhimurium transposon mutant library

S. Typhimurium was mutagenized using a Tn*5*-derived transposon as described previously (Langridge et al., 2009; a detailed protocol is available in Langridge, 2010). Briefly, the transposon was combined with the EZ-Tn*5* transposase (Epicenter, Madison, USA) and electroporated into *S.* Typhimurium. Transformants were selected by plating on LB agar containing 15 μg/mL kanamycin and harvested directly from the plates following overnight incubation. A typical electroporation experiment generated a batch of between

50,000 and 150,000 individual mutants. 10 batches were pooled together to create a mutant library comprising approximately 930,000 transposon mutants.

2.2.4 DNA manipulations and sequencing

Genomic DNA was extracted from the library pool samples using tip-100g columns and the genomic DNA buffer set from Qiagen (Crawley, UK). DNA was prepared for nucleotide sequencing as described previously (Langridge et al., 2009). Prior to sequencing, a 22 cycle PCR was performed as previously described (Langridge et al., 2009). Sequencing took place on a single end Illumina flowcell using an Illumina GAII sequencer, for 36 cycles of sequencing, using a custom sequencing primer and 2x Hybridization Buffer (Langridge et al., 2009). The custom primer was designed such that the first 10 bp of each read was transposon sequence.

2.2.5 Sequence analysis

The Illumina FASTQ sequence files were parsed for 100% identity to the 5′ 10bp of the transposon (TAAGAGACAG). Sequence reads which matched were stripped of the transposon tag and subsequently mapped to the *S.* Typhimurium SL1344 or *S.* Typhi Ty2 chromosomes using MAQ version maq-0.6.8 (Li et al., 2008). Approximately 12 million sequence reads were generated from the sequencing run which used two lanes on the Illumina flowcell. Precise insertion sites were determined using the output from the Maq mapview command, which gives the first nucleotide position to which each read mapped. The number and frequency of insertions mapping to each nucleotide in the appropriate genome was then determined.

2.2.6 Statistical analysis of required genes

The number of insertion sites for any gene is dependent upon its length, so the values were made comparable by dividing the number of insertion sites by the gene length, giving an "insertion index" for each gene. As before (Langridge et al., 2009) the distribution of insertion indices was bimodal, corresponding to the required (mode at 0) and non-required distributions (See Figure 2.2). I fitted gamma distributions for the two modes using the R MASS library (http://www.r-project.org). Log_2-likelihood ratios (LLR) were calculated

between the required and non-required distributions and I called a gene required if it had an LLR of less than -2, indicating it was at least 4 times more likely according to the required model than the non-required model. "Non-required" genes were assigned for an LLR of greater than 2. Genes falling between the two thresholds were considered "ambiguous" for the purpose of this analysis. This procedure lead to genes being called as required in *S.* Typhimurium when their insertion index was less than 0.020, or 1 insertion in every 50 bases, and ambiguous between 0.020 and 0.027. The equivalent cut-offs for the *S.* Typhi library are 0.0147 and 0.0186, respectively.

I calculated a p-value for the observed number of insertion sites per gene using a Poisson approximation with rate $R = \frac{N}{G}$ where N is the number of unique insert sites (549,086) and G is the number of bases in the genome (4,878,012). The p-value for at least X consecutive bases without an insert site is $e^{(-RX)}$, giving a 5% cut-off at 27 bp and a 1% cut-off at 41 bp.

For every gene g with $n_{g,A}$ reads observed in *S.* Typhi and $n_{g,B}$ reads observed in *S.* Typhimurium, I calculated the $\log_2$ fold change ratio $S_{g,A,B} = \log_2(\frac{n_{g,A}+100}{n_{g,B}+100})$. The correction of 100 reads smoothes out the high scores for genes with very low numbers of observed reads. I fitted a normal distribution to the mode +/- 2 sample standard deviations of the distribution of $S_{A,B}$, and calculated p-values for each gene according to the fit. I considered genes with a p-value of 0.05 or less under the fitted normal distribution to be uniquely required by one serovar.

2.3 Results and Discussion

2.3.1 TraDIS assay of every *Salmonella* Typhimurium protein-coding gene

Approximately 930,000 mutants of *S.* Typhimurium were generated using a Tn*5*-derived transposon. 549,086 unique insertion sites were recovered from the mutant library using short-read sequencing with transposon-specific primers. This is a substantially higher density than the 371,775 insertions recovered from *S.* Typhi previously (Langridge et al., 2009). The *S.* Typhimurium library contains an average of one insertion every 9bp, or over 100 unique inserts per gene (figure 2.3). The large number of unique insertion sites allowed every gene to be assayed; assuming random insertion across the genome, a region

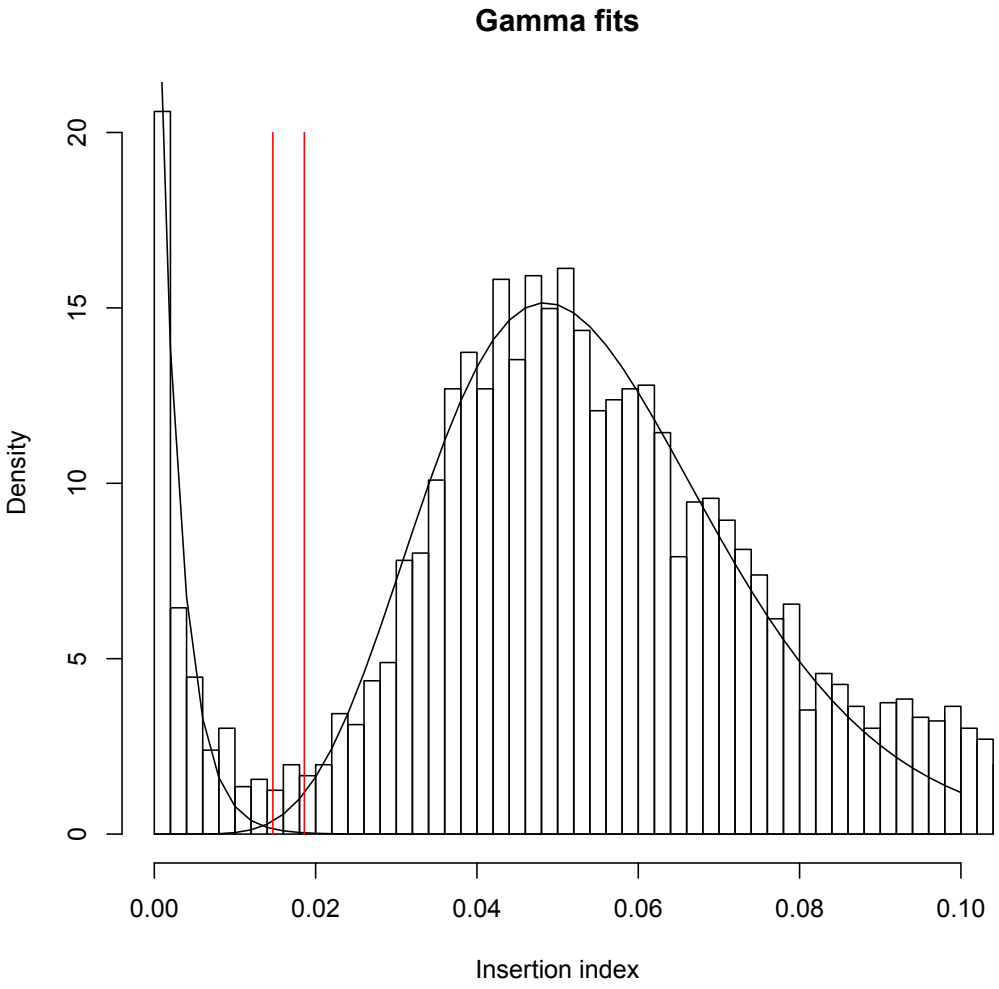


Figure 2.2: The distribution of gene-wise insertion indexes in *S.* Typhi. Bars report the density of genes with insertion indexes within each range, black lines show gamma distributions fitted to the required (left, mode at 0) and non-required (right) peaks, and red lines report associated LLR-based cut-offs for calling gene ambiguity (left) and requirement (right). The distribution of insertion indexes in *S.* Typhimurium is similar, though with a wider separation between the required and non-required peaks due to the higher insertion density attained.

of 41bp without an insertion was statistically significant ($P < 0.01$). As previously noted in *S.* Typhi, the distribution of length-normalized insertions per gene is bimodal (see figure 2.2), with one mode at 0. Genes falling in to the distribution around this mode are interpreted as being required for competitive growth within a mixed population under laboratory conditions (hereafter "required"). Of these, 57 contained no insertions whatsoever and were mostly involved in core cellular processes (see table 2.1).

There was a bias in the frequency of transposon insertion towards the origin of replication. This likely occurred as the bacteria were in exponential growth phase immediately prior to transformation with the transposon. In this phase of growth, multiple replication forks would have been initiated, meaning genes closer to the origin were in greater copy number and hence more likely to be a target for insertion. There was a bias for transposon insertions in A+T rich regions, as was previously observed in the construction of an *S.* Typhi mutant library (Langridge et al., 2009). However, the insertion density achieved is sufficient to discriminate between required and non-required genes easily. As was first seen in *S.* Typhi (Langridge et al., 2009), there were transposon insertions into genes upstream of required genes in the same operon, suggesting that most insertions do not have polar effects leading to the inactivation of downstream genes.

Analysis of the *S.* Typhimurium mutant library allowed the identification of 353 coding sequences required for growth under laboratory conditions, and 4,112 non-required coding sequences (see Appendix A for details). Sixty-five genes could not be assigned to either the required or non-required category. 60 of these genes, which I will refer to as "ambiguous", had log-likelihood ratios (LLRs) between -2 and 2. The final 5 unassigned genes had lengths less than 60 bases, and they were removed from the analysis. All other genes contained enough insertions or were of sufficient length to generate credible LLR scores. Thus, every gene was assayed and I was able to draw conclusions for 98.7% of the coding genome in a single sequencing run (figure 2.3).

2.3.2 Cross-species comparison of genes required for growth

Gene essentiality has previously been assayed in *S.* Typhimurium using insertion-duplication mutagenesis. Knuth et al. (2004) estimated 490 genes are essential to growth in clonal populations, though 36 of these have subsequently been successfully deleted (Santiviago et al., 2009). While TraDIS assays gene requirements after a brief

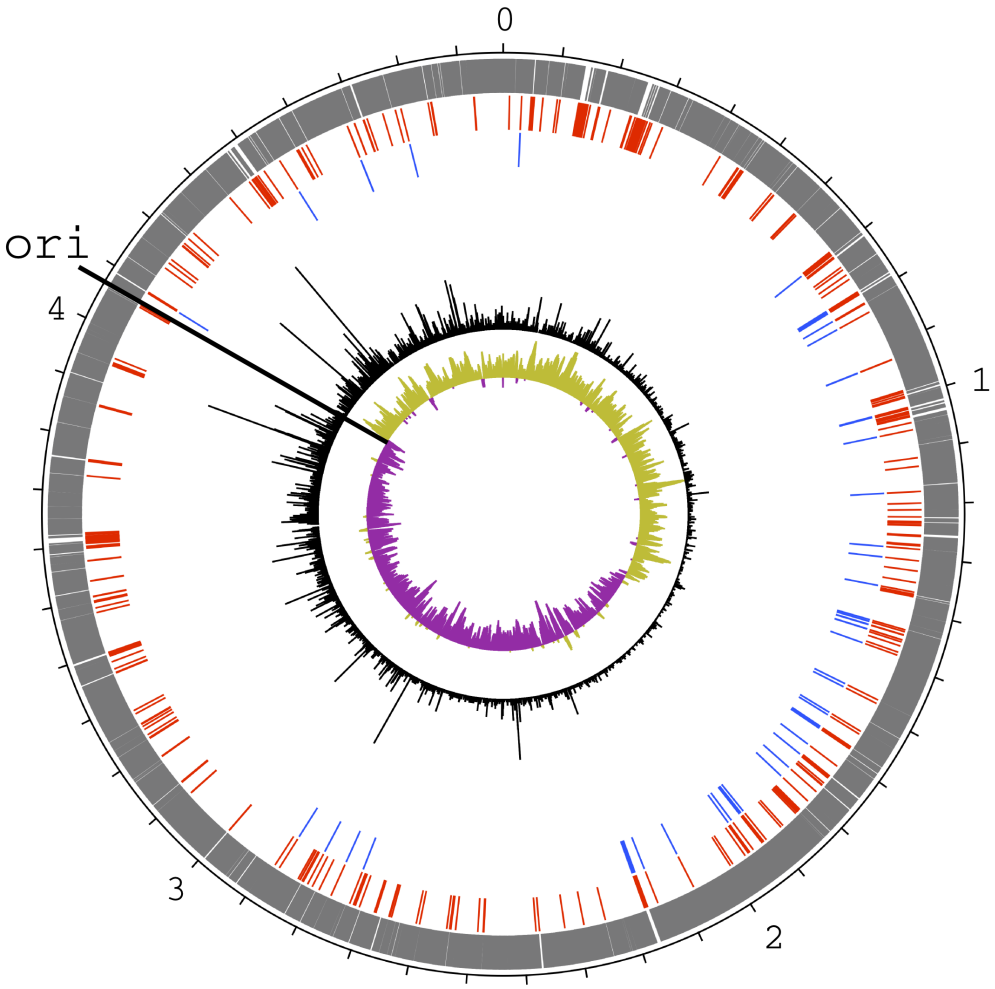


Figure 2.3: Genome-wide transposon mutagenesis of *S.* Typhimurium. Circular plot showing gene content, distribution of required genes, and insertion density along the *S.* Typhimurium chromosome. The outer scale is marked in megabases. Circular tracks range from 1 (outer track) to 5 (inner track). Track 1, Typhimurium non-required genes (grey); track 2, *S.* Typhimurium required genes (red); track 3, 56 genes required by *S.* Typhimurium but not by *S.* Typhi (dark blue, see also table 1); track 4, transposon insertion density; track 5, GC bias ($\frac{G-C}{G+C}$), yellow indicates values >1; purple <1.

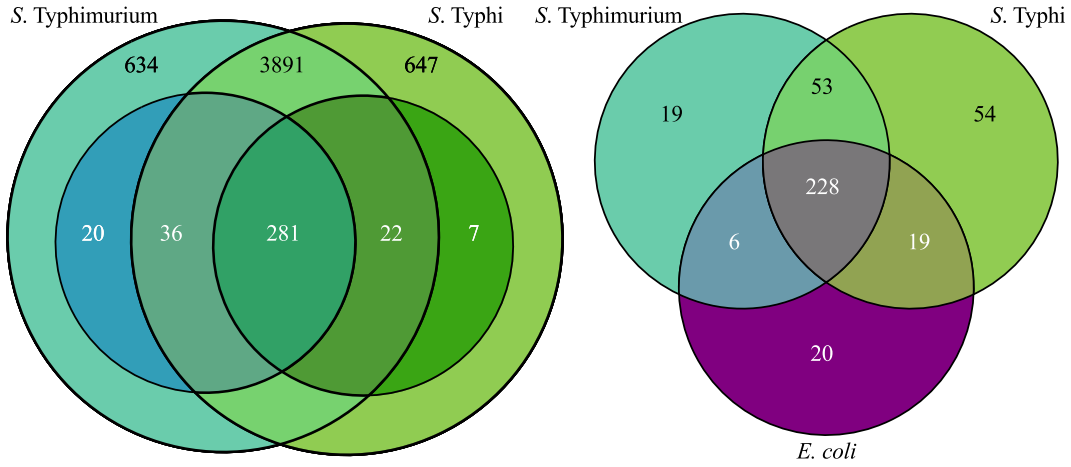


Figure 2.4: Comparison of required genes. Left, venn diagram showing the overlap of all genes (outer circles, light colors) and required genes (inner circles, dark colors) between *S.* Typhimurium and *S.* Typhi (excluding genes required in one serovar only which do not have significantly different read-counts). Black numbers refer to all genes, white numbers to required genes. Right, the overlap of all required genes between *S.* Typhimurium (blue), *S.* Typhi (green) and *E. coli* (purple). White numbers refer to genes with Keio essentiality scores >= 0.5.

period of competitive growth on rich media, I identify a smaller required set than Knuth et al. (2004) of approximately 350 genes in each serovar, closer to current estimates of approximately 300 essential genes in *E. coli* (Baba et al., 2006).

To demonstrate that TraDIS does identify genes known to have strong effects on growth, as well as to test our predictive power for determining gene essentiality, I compared our required gene sets in *S.* Typhimurium and *S.* Typhi to essential genes determined by systematic single-gene knockouts in the *Escherichia coli* K-12 Keio collection (Baba et al., 2006). I identified orthologous genes in the three data sets by best reciprocal FASTA hits exhibiting over 30% sequence identity over at least 80% of the amino acid sequence. Required orthologous genes identified in this manner share a significantly higher average percent sequence identity with their *E. coli* counterparts than expected for a random set of orthologs, at ∼94% identity as compared to ∼87% for all orthologous genes. In 100,000 randomly chosen gene sets of the same size as our required set I did not find a single set where the average shared identity exceeded 90%, indicating that required genes identified by TraDIS are more highly conserved at the amino acid level than other orthologous protein coding sequences.

Baba et al. (2006) have defined an essentiality score for each gene in *E. coli* based on evidence from four experimental techniques for determining gene essentiality: targeted knock-outs using λ-red mediated homologous recombination, genetic footprinting (Gerdes et al., 2003; Tong et al., 2004), large-scale chromosomal deletions (Hashimoto et al., 2005), and transposon mutagenesis (Kang et al., 2004). Scores range from -4 to 3, with negative scores indicating evidence for non-essentiality and positive scores indicating evidence for essentiality. Comparing the overlap between essential gene sets in *E. coli*, *S.* Typhi, and *S.* Typhimurium, I found a set of 228 *E. coli* genes which have a Keio essentiality score of at least 0.5 (i.e. there is evidence for gene essentiality; See Figure 2.4.) that have TraDIS-predicted required orthologs in both *S.* Typhi and *S.* Typhimurium, constituting ∼85% of *E. coli* genes with evidence for essentiality indicating that gene requirements are largely conserved between these genera. Including orthologous genes that are only predicted to be essential by TraDIS in *S.* Typhi or *S.* Typhimurium raises this figure to nearly 93%. The majority of shared required genes between all three bacteria are responsible for fundamental cell processes, including cell division, transcription and translation. A number of key metabolic pathways are also represented, such as fatty acid and peptidoglycan biosynthesis (Table 2.1). A recent study in the α-proteobacteria *Caulobacter crescentus* reported 210 shared essential genes with *E. coli*, despite *C. crescentus* sharing less than a third as many orthologous genes with *E. coli* as *Salmonella* serovars (Christen et al., 2011). This suggests the existence of a shared core of approximately 200 essential proteobacterial genes, with the comparatively rapid turnover of 150 to 250 non-core lineage-specific essential genes.

Table 2.1: Core genome functions in *S.* Typhimurium. Protein-coding genes providing fundamental biological functions in *S.* Typhimurium. Genes in bold are required in*S.* Typhi (log-likelihood ratio (LLR) between required and non-required models < -2; see Methods.) * indicates genes ambiguous in *S.* Typhimurium, having a LLR between -2 and 2.

Biological Process	Sub-process	Required genes	Non-required genes
Cell division		***ftsALKQWYZ***, *minE, mukB, SL2391*	***ftsHJNX****, *minCD, sdiA, cedA, sulA*
DNA replications	Polymerases I, II, and III	***dnaENQX, holAB***	***polA****B, holC****DE***
	Supercoiling	***gyrAB, parCE***	
	Primosome-associated	***dnaBCGT, priA, ssb***	***priB****C, **rep***
Transcription	RNA polymerase	***rpoABC***	
	Sigma, elongation, anti- and termination factors	***nusBG, rpoDH, rho***	***nusA, rpoE****NS*
Translation	tRNA-synthetases	***alaS, argS, asnS, aspS, cysS, glnS, gltX, glyQS, hisS, ileS, leuS, lysS, metG, pheST, proS, serS, thrS, tyrS, valS***	*trpS, trpS2*
	Ribosome components	***rplBCDEFJKLMNOPQRSTUVWXY, rpmABCDHI, rpsABCDEFGHIJKLMNPQST***	*rplAI, rpmEE2, rpmFHJJ2, rps****OR****U****V*
	Initiation, elongation, and peptide chain release factors	***fusA, infABC, prfAB, tsf, yrdC***	*efp, prfCH, selB, tuf*
Biosynthetic pathways			
Peptidoglycan		***murABCDEFGI***	*ddl, dllA*
Fatty acids		***accABCD, fabABDGHIZ***	

By making the simplistic assumption that gene essentiality should be conserved between *E. coli* and *Salmonella*, I can use the overlap of our predictions with the Keio essential genes to provide an estimate of our TraDIS libraries accuracy for predicting that a gene will be required in a clonal population. Of the 2632 orthologous *E. coli* genes which have a Keio essentiality score of less than -0.5 (i.e. there is evidence for gene non-essentiality), only 33 are predicted to be required by TraDIS in both *Salmonella* serovars. *S.* Typhi contains the largest number of genes predicted by TraDIS to be required with *E. coli* orthologs with negative Keio essentiality scores. However, even if it is assumed these are all incorrect predictions of gene essentiality, this still gives a gene-wise false positive rate (FPR) of ∼2.7% (81 out of 2981 orthologs) and a positive predictive value (PPV) of ∼75% (247 with essentiality scores greater than or equal to 0.5 out of 328 predictions with some Keio essentiality score.) Under these same criteria the *S.* Typhimurium data set has a lower gene-wise FPR of ∼1.6% (51 out of 3122 orthologs) and a higher PPV of ∼82% (234 out of 285 predictions as before), as would be expected given the library's higher insertion density. In reality these FPRs and PPVs are only estimates; genes which are not essential in *E. coli* may become essential in the different genomic context of *Salmonella* serovars and vice versa, particularly in the case of *S.* Typhi where wide-spread pseudogene formation has eliminated potentially redundant pathways (Holt et al., 2009; McClelland et al., 2004). Additionally, TraDIS will naturally over-predict essentiality in comparison to targeted knockouts, as the library creation protocol necessarily contains a short period of competitive growth between mutants during the recovery from electro-transformation and selection. As a consequence, genes which cause major growth defects, but not necessarily a complete lack of viability in clonal populations, may be reported as 'required.'

2.3.3 Serovar-specific genes required for growth

Many of the required genes present in only one serovar encoded phage repressors, for instance the cI proteins of Fels-2/SopE and ST35 (see tables 2.2 and 2.3). Repressors maintain the lysogenic state of prophage, preventing transcription of early lytic genes (Echols, 1971). Transposon insertions into these genes will relieve this repression and trigger the lytic cycle, resulting in cell death, and consequently mutants are not represented in the sequenced library. This again broadens the definition of 'required' genes; such

Table 2.2: Phage elements in *S.* Typhimurium. Genomic coordinates determined from annotations in the EMBL annotation for FQ312003 and manual inspection. Repressor domains and architecture were determined using the HMMER webserver (Finn et al., 2011) and Pfam (Punta et al., 2012). Phage types were determined using repressor sequence similarity searches and information from Thomson et al. (2004) and Kropinski et al. (2007).

Element name	Genomic coordinates	Repressor	Repressor domain(s)	Repressor domain architecture	Predicted active?	Phage type	Required cargo
Gifsy-2 SLP105	1054795 - 1100036	SL0950	HTH_3 (PF01381)		Yes	lambdoid	N/A
N/A	1913364 - 1925490	N/A	N/A	N/A	No	remnant	SL1799
SLP203	2039803 - 2079890	SL1967	HTH_19 (PF12844) and Peptidase_S24 (PF00717)		Yes	P22-like	N/A
Gifsy-1 SLP272	2726717 - 2777229	SL2593	HTH_3 (PF01381)		Yes	lambdoid	SL2549
SLP281	2815382 - 2825915	SL2633	2 X Phage_CI_repr (PF07022)		Yes	degenerate P2-like	N/A
Fels-2 SLP285	2855616 - 2888522	SL2708	Phage_CI_repr (PF07022)		Yes	P2-like	SL2695
SLP289	2890073 - 2900377	IsrK RNA (RF01394)	N/A	N/A	No	P4-like	N/A
SLP443	4437731 - 4459844	N/A	N/A	N/A	No	remnant	SL4132

repressors may not be required for cellular viability in the traditional sense, but once present in these particular genomes, their maintenance is required for continued viability, as long as the rest of the phage remains intact.

S. Typhimurium and *S.* Typhi both contain 8 apparent large phage-derived genomic regions (Thomson et al., 2004; Kropinski et al., 2007). I was able to identify required repressors in all the intact lambdoid, P2-like, and P22-like prophage in both genomes, including Gifsy-1, Gifsy-2, and Fels-2/SopE (see tables 2.2 and 2.3). With the exception of the SLP203 P22-like prophage in *S.* Typhimurium, all of these repressors lack the peptidase domain of the classical λ repressor gene cI. This implies that the default anti-repression mechanism of *Salmonella* prophage may be more similar to a trans-acting mechanism recently discovered in Gifsy phage (Lemire et al., 2011) than to the λ repressor's RecA-induced self-cleavage mechanism. I was also able to confirm that most phage remnants and fusions contained no active repressors, with the exception of the SLP281 degenerate P2-like prophage in *S.* Typhimurium. This degenerate prophage contains both intact replication and integration genes, but appears to lack tail and head proteins, suggesting it may depend on another phage for production of viral particles.

Table 2.3: Phage elements in *S.* Typhi. Genomic coordinates determined from Thomson et al. (2004) and manual inspection. Repressor domains and architecture were determined using the HMMER webserver (Finn et al., 2011) and Pfam (Punta et al., 2012). Phage types were determined using repressor sequence similarity searches and information from Thomson et al. (2004) and Kropinski et al. (2007).

Element name	Genomic coordinates	Repressor	Repressor domain(s)	Repressor domain architecture	Predicted active?	Phage type	Required cargo
ST15	1408790 - 1441377	N/A	N/A	N/A	No	Mu/P2 fusion	N/A
Gifsy-2	1929572 - 1972330	t1920	HTH_3 (PF01381)		Yes	lambdoid	N/A
ST2-27	2735054 - 2745321	IsrK RNA (RF01394)	N/A	N/A	Yes	P4-like	N/A
ST27	2745477 - 2768221	N/A	N/A	N/A	No	P2/iroA fusion	N/A
ST35	3500854 - 3536047	t3402	Phage_CI_repr (PF07022)		Yes	P2-like	t3415
SopE	4457346 - 4491316	t4337	Phage_CI_repr (PF07022)		Yes	P2-like	N/A
N/A	4519423 - 4519501	IsrK RNA (RF01394)	N/A	N/A	No	remnant	N/A
ST46	4666579 - 4677433	IsrK RNA (RF01394)	N/A	N/A	Yes	P4-like	N/A

Both genomes also encode P4-like satellite prophage, which rely on 'helper' phage for lytic functions and utilize a complex antisense-RNA based regulation mechanism for decision pathways regarding cell fate (Briani et al., 2001) using structural homologs of the IsrK (Padalon-Brauch et al., 2008) and C4 ncRNAs (Forti et al., 2002), known as seqA and CI RNA in the P4 literature, respectively. While the mechanism of P4 lysogenic maintenance is not known, the IsrK-like ncRNAs of two potentially active P4-like prophage in *S.* Typhi are required under TraDIS. This sequence element has previously been shown to be essential for the establishment of the P4 lysogenic state (Sabbattini et al., 1995), and we predict based on our observations that it may be necessary for lysogenic maintenance as well. The fact that some lambdoid prophage in *S.* Typhimurium encode non-coding genes structurally similar to the IsrK-C4 immunity system of P4 raises the possibility that these systems may be acting as a defense mechanism of sorts, protecting the prophage from predatory satellite phage capable of co-opting its lytic genes.

In addition to repressors, 4 prophage cargo genes in *S.* Typhimurium and one in *S.* Typhi are required (See tables 2.2, 2.3, 2.4, and 2.6). The *S.* Typhimurium prophage cargo genes encode a PhoPQ regulated protein, a protein predicted to be involved in natural transformation, an endodeoxyribonuclease, and a hypothetical protein. The

S. Typhi prophage cargo gene encodes a protein containing the DNA-binding HIRAN domain (Iyer et al., 2006), believed to be involved in the repair of damaged DNA. These warrant further investigation, as they are genes that have been recently acquired and become necessary for survival in rich media.

To compare differences between requirements for orthologous genes in both serovars, I calculated log-fold read ratios to eliminate genes which were classified differently in *S.* Typhi and *S.* Typhimurium but did not have significantly different read densities (see Methods.) Even after this correction, 36 *S.* Typhimurium genes had a significantly lower frequency of transposon insertion compared to the equivalent genes in *S.* Typhi ($P < 0.05$), including four encoding hypothetical proteins (table 2.4). This indicates that these gene products play a vital role in *S.* Typhimurium but not in *S.* Typhi when grown under laboratory conditions.

Table 2.4: Genes uniquely required in *S.* Typhimurium. Genes determined to be uniquely required in *S.* Typhimurium. SL, *S.* Typhimurium; Ty, *S.* Typhi; inserts refer to the number of unique insertion sites within a gene; reads refer to the number of sequence reads over all insertions sites within a gene. †, P-value (associated with log2 read ratio) < 0.05. ‡, *sseJ* is a pseudogene in S. Typhi. Shaded rows indicate genes shown to be H-NS repressed in Navarre et al. (2006)

	Ty inserts	Ty reads	SL inserts	SL reads	SL ID	SL gene length	Ty ID	Ty gene length	Name	Function
No ortholog in *S.* Typhi	-	-	18	123	SL0742	1269	-	-	-	putative cation transporter
	-	-	9	80	SL0830	516	-	-	-	conserved hypothetical protein
	-	-	4	21	SL0831	855	-	-	-	putative electron transfer flavoprotein (beta subunit)
	-	-	0	0	SL0950	323	-	-	-	predicted bacteriophage protein, potential phage repressor Gifsy-2
	-	-	11	75	SL1179	789	-	-	envF	lipoprotein
	-	-	3	18	SL1480	249	-	-	-	antitoxin Phd_YefM, type II toxin-antitoxin system
	-	-	4	32	SL1527	264	-	-	ydcX	putative inner membrane protein
	-	-	1	3	SL1560	717	-	-	-	putative membrane protein
	-	-	7	50	SL1601	859	-	-	-	putative transcriptional regulator (pseudogene)
	-	-	4	36	SL1799	201	-	-	-	bacteriophage encoded pagK (phoPQ-activated protein)
	-	-	5	22	SL1830A	434	-	-	-	conserved hypothetical protein (pseudogene)
	-	-	3	27	SL1967	677	-	-	-	predicted bacteriophage protein, potential phage repressor SLP203
	-	-	1	15	SL2045A	63	-	-	yoeI	short ORF
	-	-	17	107	SL2066	900	-	-	rfbJ	CDP-abequose synthase
	-	-	3	34	SL2549	209	-	-	-	endodeoxyribonuclease
	-	-	4	149	SL2593	449	-	-	-	putative DNA-binding protein, potential phage repressor Gifsy-1 SLP272
	-	-	3	7	SL2633	846	-	-	-	putative repressor protein, phage SLP281
	-	-	2	21	SL2695	978	-	-	smf	putative competence protein
	-	-	5	39	SL4132	291	-	-	-	hypothetical protein
	-	-	5	45	SL4354A	303	-	-	-	conserved hypothetical protein
Present in *S.* Typhi but required only in *S.* Typhimurium†	36	474	5	26	SL0032	441	t0033	306	-	putative transcriptional regulator
	71	349	11	48	SL0623	642	t2232	576	lipB	lipoate-protein ligase B
	151	3546	10	64	SL0702	897	t2156	894	-	putative glycosyl transferase
	194	3007	9	61	SL0703	1134	t2155	1134	-	galactosyltransferase
	231	3499	15	67	SL0706	1779	t2152	1780	-	putative glycosyltransferase, cell wall biogenesis
	84	1041	2	4	SL0707	834	t2151	834	-	putative glycosyltransferase, cell wall biogenesis
	49	367	14	70	SL0722	1569	t2136	1569	cydA	cytochrome d ubiquinol oxidase subunit I
	74	1613	5	22	SL1069	693	t1789	693	-	putative secreted protein
	20	199	1	1	SL1203	150	t1146	156	-	hypothetical protein

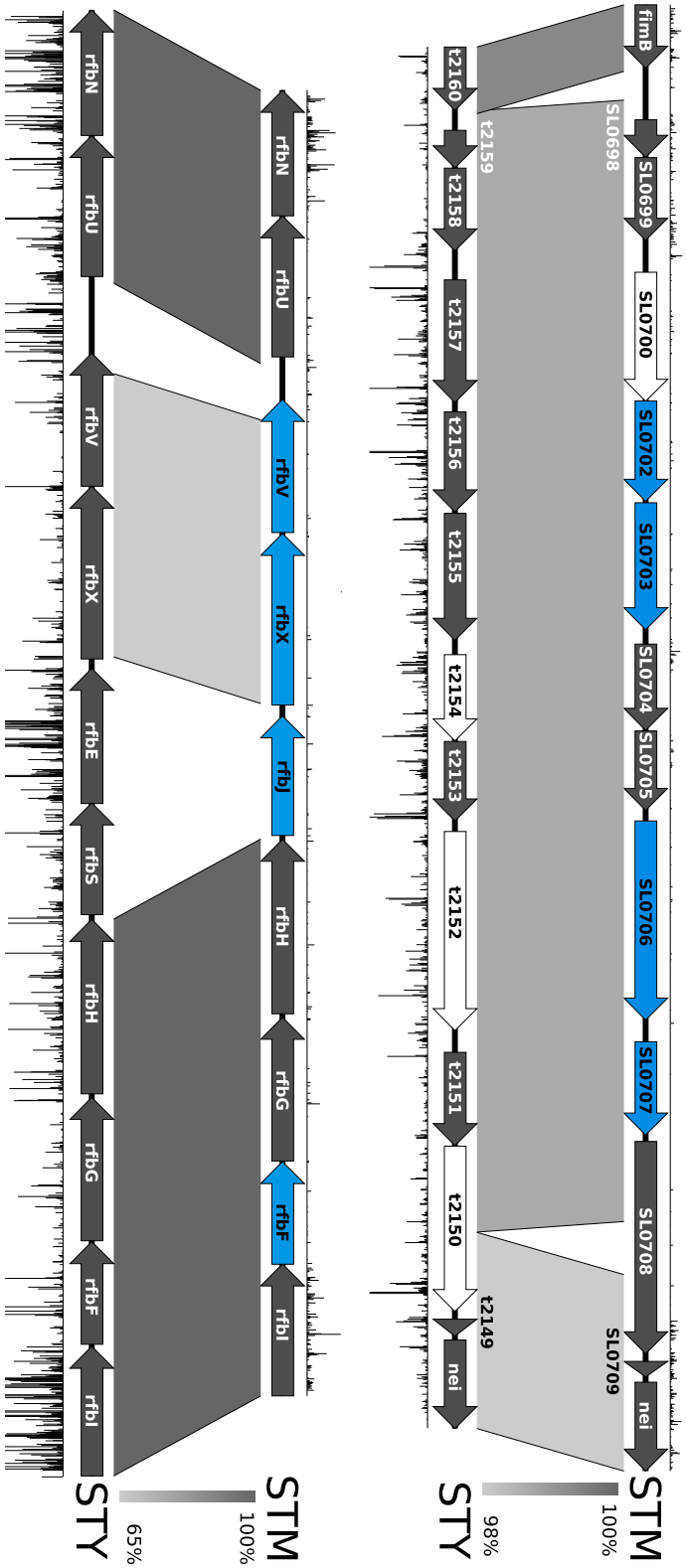
Present in *S.* Typhi but required only in *S.* Typhimurium

20	290	1	5	SL1264	315	t1209	315	-		putative membrane protein
84	384	6	26	SL1327	402	t1261	384	spiC		putative pathogenicity island 2 secreted effector protein
66	769	5	35	SL1331	270	t1265	327	sseA		T3SS chaperone
36	307	2	5	SL1341	228	t1275	228	ssaH		putative pathogenicity island protein
47	407	1	3	SL1342	249	t1276	249	ssaI		putative pathogenicity island protein
144	3197	5	14	SL1343	750	t1277	750	ssaJ		putative pathogenicity island lipoprotein
63	847	5	26	SL1354	267	t1288	267	ssaS		putative type III secretion protein
73	762	4	44	SL1355	780	t1289	780	ssaT		putative type III secretion protein
30	226	12	48	SL1386	693	t1322	693	rnfE/ydgQ		Electron transport complex protein rnfE
265	3337	29	165	SL1473	1557	t1463	1557	pqaA		PhoPQ-activated protein
85	765	6	35	SL1532	951	t1511	951	sifB		putative virulence effector protein
22	156	16	174	SL1561	1227	t1534‡	141	sseJ		Salmonella translocated effector protein (SseJ)
119	1639	10	44	SL1563	762	t1536	762	-		putative periplasmic amino acid-binding protein
107	2440	5	44	SL1564	648	t1537	648	-		putative ABC amino acid transporter permease
183	1646	20	118	SL1628	1355	t1612	1364	-		hypothetical protein
23	177	1	5	SL1659	183	t1640	183	yciG		conserved hypothetical protein
78	617	16	104	SL1684	1014	t1664	1014	hnr		putative regulatory protein
37	277	4	25	SL1785	396	t1022	396	-		conserved hypothetical protein
166	2823	9	27	SL1793	915	t1016	915	pagO		inner membrane protein
28	311	3	22	SL1794	159	t1015	159	-		putative inner membrane protein
23	155	1	4	SL1823	972	t0988	972	msbB		lipid A acyltransferase
60	402	11	58	SL2064	1002	t0786	1002	rfbV		putative glycosyl transferase
87	524	7	59	SL2065	1293	t0785	1299	rfbX		putative O-antigen transporter
66	559	13	74	SL2069	774	t0780	774	rfbF		glucose-1-phosphate cytidylyltransferase
41	204	5	14	SL3828	1830	t3658	1830	glmS		glucosamine-fructose-6-phosphate aminotransferase
27	288	5	23	SL4250	288	t4220	288	-		putative GerE family regulatory protein
148	2633	16	89	SL4251	876	t4221	876	-		araC family regulatory protein

A major difference between the two serovars is in the requirement for genes involved in cell wall biosynthesis (see figure 2.5). A set of four genes (SL0702, SL0703, SL0706, and SL0707) in an operonic structure putatively involved in cell wall biogenesis is required in *S.* Typhimurium but not in *S.* Typhi. The protein encoded by SL0706 is a pseudogene in *S.* Typhi (Ty2 unique ID: t2152) due to a 1bp deletion at codon 62 that causes a frameshift. This operon contains an additional two pseudogenes in *S.* Typhi (t2154 and t2150), as well as a single different pseudogene (SL0700) in *S.* Typhimurium, indicating that this difference in gene requirements reflects the evolutionary adaptation of these serovars to their respective niches. Similarly, four genes (*rfbV*, *rfbX*, *rfbJ* and *rfbF*) within an O-antigen biosynthetic operon are required by *S.* Typhimurium but not *S.* Typhi. There appears to have been a shuffling of O-antigen biosynthetic genes since the divergence between the two serovars, and *rfbJ*, encoding a CDP-abequose synthase, has been lost from *S.* Typhi altogether. These broader requirements for cell wall-associated biosynthetic and transporter genes suggest that surface structure biogenesis is of greater importance in *S.* Typhimurium.

There were seven genes from the shared pathogenicity island SPI-2 that appear to contain few or no transposon insertions only in *S.* Typhimurium under laboratory conditions. These genes (*spiC*, *sseA*, and *ssaHIJT*) are thought to encode components of the SPI-2 type III secretion system apparatus (T3SS) (Kuhle et al., 2004). In addition, the effector genes *sseJ* and *sifB*, whose products are secreted through the SPI-2-encoded T3SS (Miao et al., 2000; Freeman et al., 2003), also fell into the 'required' category in *S.* Typhimurium alone. All of these genes display high A+T nucleotide sequence and have been previously shown (in *S.* Typhimurium) to be strongly bound by the nucleoid associated protein H-NS, encoded by *hns* (Lucchini et al., 2006; Navarre et al.,

Figure 2.5 *(following page)*: Comparison of cell surface operon structure and requirements. Diagram illustrating cell surface operons with different requirement patterns in *S.* Typhimurium and *S.* Typhi. The top figure is of an uncharacterized operon putatively involved in cell wall biogenesis, while the bottom figure shows a portion of the *rfb* operon involved in O-antigen biosynthesis. Plots along the top and bottom of each figure show insertions in *S.* Typhimurium and *S.* Typhi, respectively, with read depth on the y-axis with a maximum cut-off of 100 reads. Genes in blue are required in *S.* Typhimurium, genes in white are pseudogenes; others are in grey. Grey rectangles represent BLAST hits between orthologous genes, with percent nucleotide identity colored on the scale to the right of each figure.


fimB
SL0698
SL0699
SL0700
SL0702
SL0703
SL0704
SL0705
SL0706
SL0707
SL0708
SL0709
nei
t2160
t2159
t2158
t2157
t2156
t2155
t2154
t2153
t2152
t2151
t2150
t2149
nei
STM
100%
STY
98%
rfbN
rfbU
rfbV
rfbX
rfbE
rfbS
rfbH
rfbG
rfbF
rfbI
rfbN
rfbU
rfbV
rfbX
rfbJ
rfbH
rfbG
rfbF
rfbI
STM
100%
STY
65%

2006). Therefore, rather than being 'required', it is instead possible that access for the transposon was sufficiently restricted that very few insertions occurred at these sites. In further support of this hypothesis, a comparison of the binding pattern of H-NS detected in studies using *S.* Typhimurium LT2 with the TraDIS results from the SPI-2 locus indicated that high regions of H-NS enrichment correlated well with both the *ssa* genes described here and with *sseJ* (see figure 2.6). An earlier study also suggests that high-density DNA binding proteins can block Mu, Tn5, and Tn10 insertion (Manna et al., 2007); however, a genome-wide study of the effects of H-NS binding on transposition would be necessary to confirm this effect.

Indeed, the generation of null *S.* Typhimurium mutants in *sseJ* and *sifB*, as well as many others generated at the SPI-2 locus suggest that these genes are not truly a requirement for growth in this serovar (Freeman et al., 2003; Hensel et al., 1997; Hensel et al., 1998; Ohlson et al., 2005). While this is a reminder that the interpretation of gene requirement needs to be made with care, the effect of H-NS upon transposon insertion is not genome-wide. If this were the case, there would be an under-representation of transposon mutants in high A+T regions (known for H-NS binding), which is not what was observed. In total, only 21 required genes fall into the '*hns*-repressed' category described in Navarre et al. (2006)(see table 2.5); the remainder (almost 400) contained sufficient transposon insertions to conclude they were non-required. In addition, all SPI-1 genes that encode another T3SS and are of high A+T content were also found to be non-required. This phenomenon was not observed in *S.* Typhi, possibly because the strain used harbors the pHCM1 plasmid, which encodes the H-NS-like protein *sfh* and has been shown to affect H-NS binding (Doyle et al., 2007; Dillon et al., 2010).

Twenty-two *S.* Typhi genes had a significantly lower frequency of transposon insertion compared to orthologs in *S.* Typhimurium ($P < 0.05$), indicating that they are required only in *S.* Typhi for growth under laboratory conditions (table 2.6), including the *fepBDGC* operon. This indicates a requirement for ferric (Fe(III)) rather than ferrous

Figure 2.6 *(following page)*: H-NS enrichment across the SPI-2 locus. Based on data from Lucchini et al. (2006) where a 2 fold enrichment of H-NS-bound DNA over a total genomic DNA control in a ChIP-on-chip experiment was taken to indicate regions of H-NS binding in *S.* Typhimurium strain LT2. Assuming these binding patterns are similar in the *S.* Typhimurium strain tested in this study, H-NS binding may have affected transposon access to genes in the SPI-2 locus.

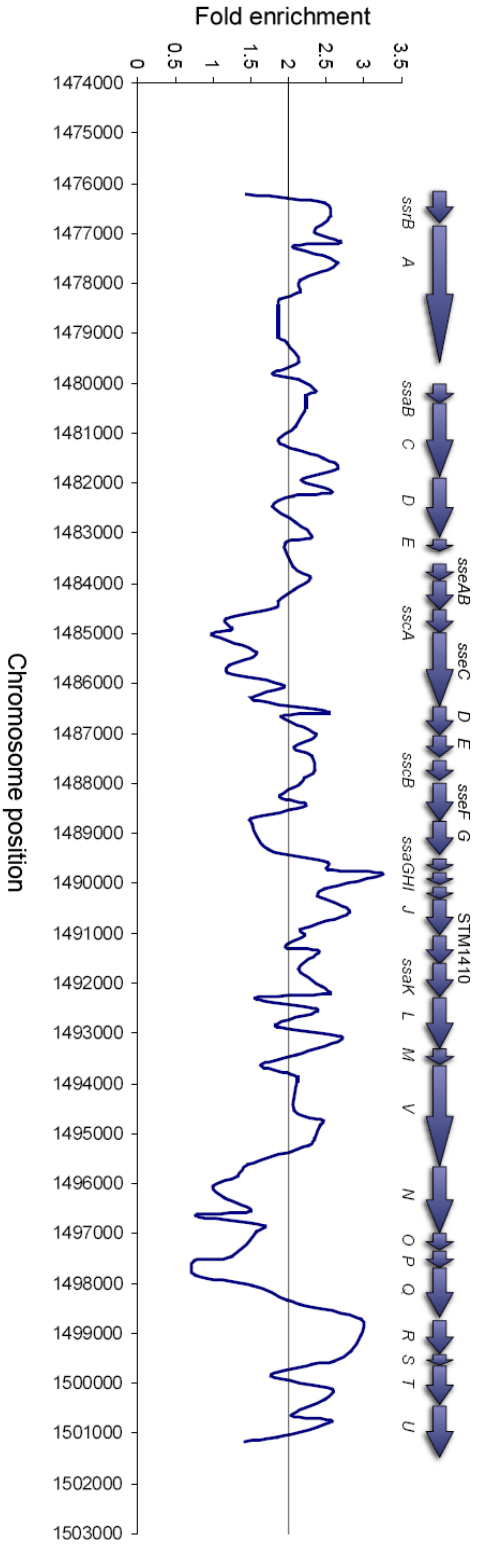

Fold enrichment
0
0.5
1
1.5
2
2.5
3
3.5
Chromosome position
1474000
1475000
1476000
1477000
1478000
1479000
1480000
1481000
1482000
1483000
1484000
1485000
1486000
1487000
1488000
1489000
1490000
1491000
1492000
1493000
1494000
1495000
1496000
1497000
1498000
1499000
1500000
1501000
1502000
1503000
ssrB
A
ssaB
C
D
E
sseAB
sscA
sseC
D
E
sscB
sseF
G
ssaGHI
J
STM1410
ssaK
L
M
V
N
O
P
Q
R
S
T
U

Table 2.5: Candidate required genes affected by H-NS binding in *S.* Typhimurium. Genes identified by comparison with data from Navarre et al. (2006). Fold change values report the results of a ChIP-on-chip experiment, and indicate genes strongly bound by H-NS.

Gene	SL ID	STM ID	Fold change	Function
-	SL0830	STM0854	-16.2	conserved hypothetical protein
-	SL0831	STM0855	-33.8	putative putative electron transfer flavoprotein (beta subunit)
-	SL1069	STM1131	-13.5	putative putative secreted protein
spiC	SL1327	STM1393	-19.1	putative pathogenicity island 2 secreted effector protein
sseA	SL1331	STM1397	-46	Type three secretion system chaperone
ssaH	SL1341	STM1407	-8.8	Type three secretion system apparatus
ssaI	SL1342	STM1408	-32.4	putative putative pathogenicity island protein
ssaJ	SL1343	STM1409	-53.7	putative putative pathogenicity island lipoprotein
ssaS	SL1354	STM1420	-15.5	putative putative type III secretion protein
ssaT	SL1355	STM1421	-33.9	putative putative type III secretion protein
pqaA	SL1473	STM1544	-5.5	PhoPQ-activated protein
sifB	SL1532	STM1602	-66.8	putative putative virulence effector protein
-	SL1560	STM1630	-9.8	putative putative membrane protein
sseJ	SL1561	STM1631	-48.6	*Salmonella* translocated effector protein (SseJ)
-	SL1563	STM1633	-91.9	putative putative periplasmic amino acid-binding protein
-	SL1564	STM1634	-22.5	putative putative ABC amino acid transporter permease
-	SL1628	STM1698	-101.4	hypothetical protein
-	SL1659	STM1728	-17.3	cytochrome b561 (cytochrome b-561)
-	SL1785	STM1856	-12.1	conserved hypothetical protein
pagO	SL1793	STM1862	-11.9	inner membrane protein (PagO)
-	SL1794	STM1864	-22.9	putative inner membrane protein

(Fe(II)) iron. This can be explained by the presence of Fe(III) in the bloodstream, where *S.* Typhi can be found during typhoid fever (Wain et al., 1998). These genes function to recover the ferric chelator enterobactin from the periplasm, acting with a number of proteins known to aid the passage of this siderophore through the outer membrane (Rabsch et al., 1999). It has long been noted that *aroA* mutants of *S.* Typhi, deficient in their ability to synthesize enterobactin, exhibit severe growth defects on complex media, while similar mutants of *S.* Typhimurium grow normally under the same conditions (Edwards et al., 1988), though the mechanism has not been clear. These results suggest that this difference in growth of *aroA* mutants is caused by a requirement for iron uptake through the *fep* system in *S.* Typhi. During host adaptation, *S.* Typhi has accumulated pseudogenes in many iron transport and response systems (McClelland et al., 2004), presumably because they are not necessary for survival in the niche *S.* Typhi occupies in the human host, which may have led to this dependence on *fep* genes. In contrast, *S.* Typhimurium generally causes intestinal rather than systemic infection and is able to utilize a wider range of iron sources, including Fe(II), a soluble form of iron present under anaerobic conditions such as those found in the intestine (Tsolis et al., 1996).

Table 2.6: Genes uniquely required in *S.* Typhi. Genes determined to be uniquely required in *S.* Typhi. SL, *S.* Typhimurium; Ty, *S.* Typhi; inserts refer to the number of unique insertion sites within a gene; reads refer to the number of sequence reads over all insertions sites within a gene. †, P-value (associated with log2 read ratio) < 0.05. *, the assigment of recA as a required gene has been described previously (Langridge et al., 2009), but briefly is believed to be due to the presence of the priC pseudogene in Typhi.

	SL inserts	SL reads	Ty inserts	Ty reads	Ty ID	Ty gene length	SL ID	SL gene length	Name	Function
No ortholog in *S.* Typhimurium	-	-	-	1	t1332	132	-	-	malY	pseudogene
	-	-	-	2	t1920	405	-	-	-	possible repressor protein, prophage 10/Gifsy-2
	-	-	-	2	t3157	165	-	-	-	conserved hypothetical protein
	-	-	-	2	t3166	228	-	-	-	spurious ORF annotation overlapping the RnaseP/M1 RNA
	-	-	-	6	t3402	570	-	-	cI	repressor protein, cs 73 prophage
	-	-	-	4	t3415	741	-	-	-	HIRAN-domain family gene, potential DNA repair
	-	-	-	1	t4531	150	-	-	-	hypothetical secreted protein
Present in *S.* Typhimurium but required only in *S.* Typhi†	199	1792	18	59	t0095	1287	SL0093	1287	surA	survival protein SurA precursor
	45	498	3	22	t0123	459	SL0119	459	yabB/mraZ	conserved hypothetical protein
	120	589	11	32	t0203	1281	SL0203	1281	hemL	glutamate-1-semialdehyde 2,1-aminomutase
	123	982	2	25	t0224	1353	SL0224	1353	yaeL/rseP	Zinc metallopeptidase
	67	452	1	14	t0270	576	SL2604	576	rpoE	RNA polymerase sigma-E factor
	140	760	0	0	t0587	2286	SL2246	2286	nrdA	ribonucleoside-diphosphate reductase 1 alpha chain
	113	641	15	42	t2140	2802	SL0718	2802	sucA	2-oxoglutarate dehydrogenase E1 component
	116	753	13	36	t2177	1641	SL0680	1641	pgm	phosphoglucomutase
	80	542	9	15	t2276	1008	SL0580	1008	fepD	ferric enterobactin transport protein FepD
	93	591	2	2	t2277	990	SL0579	990	fepG	ferric enterobactin transport protein FepG
	64	508	5	6	t2278	795	SL0578	795	fepC	ferric enterobactin transport ATP-binding protein FepC
	201	1129	12	116	t2410	2355	SL0444	2355	lon	Lon protease
	95	518	8	20	t2730	1062	SL2809	1062	recA*	recA protein
	135	719	16	39	t2996	1992	SL3052	1947	tktA	transketolase
	76	358	3	9	t3120	1434	SL3173	1434	rfaE	ADP-heptose synthase
	213	1976	6	50	t3265	1071	SL3321	1071	degS	serine protease
	43	448	3	10	t3326	606	SL3925	606	yigP	conserved hypothetical protein
	124	571	17	36	t3384	2025	SL3872	2025	rep	ATP-dependent DNA helicase
	175	1208	6	21	t3621	2787	SL3947	2787	polA	DNA polymerase I
	117	797	9	13	t3808	1047	SL3677	1047	waaF	ADP-heptose-LPS heptosyltransferase II
	176	1628	14	59	t4153	1080	SL4183	1080	alr	alanine racemase
	140	1127	10	38	t4411	951	SL4294	951	miaA	tRNA delta-2-isopentenylpyrophosphate transferase

2.3.4 TraDIS provides resolution sufficient to evaluate ncRNA contributions to fitness

Under a Poisson approximation to the transposon insertion process, a region of 41 (in *S.* Typhimurium) or 60 bases (in *S.* Typhi) has only a 1% probability of not containing an insertion. NcRNAs tend to be considerably shorter than their protein-coding counterparts, but this gives us sufficient resolution to assay most of the non-coding complement of the *Salmonella* genome. As a proof of principle, I performed an analysis of the best-understood class of small ncRNAs, the tRNAs. Francis Crick hypothesized that a single tRNA could recognize more than one codon through wobble recognition (Crick, 1966), where a non-canonical G-U base pair is formed between the first (wobble) position of the anticodon and the third nucleotide in the codon. As a result, some codons are covered by multiple tRNAs, while others are covered non-redundantly by a single tRNA. I expect that singleton wobble-capable tRNAs, that is wobble tRNAs which recognize a codon uniquely, will be required. In addition, I inferred the requirement for other tRNAs through the non-redundant coverage of their codons and used this to benchmark our ability to use TraDIS to reliably interrogate short genomic intervals.

The *S.* Typhi and *S.* Typhimurium genomes encode 78 and 85 (plus one pseudogene) tRNAs respectively with 40 anticodons, as identified by tRNAscan-SE (Lowe et al., 1997). In *S.* Typhi, 10 out of 11 singleton wobble tRNAs are predicted to be required or ambiguous, compared to 16 tRNAs below the ambiguous LLR cut-off overall (significant enrichment at the 0.05 level, two-tailed Fishers exact test p-value: 6.4e-08.) Similarly in *S.* Typhimurium, 9 of 11 singleton wobble tRNAs are required or ambiguous compared to 15 required or ambiguous tRNAs overall, again showing a significant enrichment of required tRNAs in this subset (Fishers exact test p-value: 5.2e-07.) The one singleton wobble tRNA which is consistently not required in both serovars is the tRNA-Pro(GGG), which occurs within a 4-member codon family. It has previously been shown in *S.* Typhimurium that tRNA-Pro(UGG) can read all four proline codons in vivo due to a cmo5U34 modification to the anticodon, obviating the need for a functional tRNA-Pro(GGG) (Näsvall et al., 2004) and making this tRNA non-required. The other non-required singleton wobble tRNA in *S.* Typhimurium, tRNA-Leu(GAG), is similarly a member of a 4-member codon family. I predict tRNA-Leu(TAG) may also be capable of recognizing all 4 leucine codons in this serovar; such a leucine "four-way wobble" has been previously inferred in at least

one other bacterial species (Osawa et al., 1992; Marck et al., 2002).

Of the 6 required non-wobble tRNAs in each serovar, four are shared. These include two non-wobble singleton tRNAs covering codons uniquely, as well as a tRNA with the ATG anticodon which is post-transcriptionally modified by the required protein MesJ/TilS to recognize the isoleucine codon ATA (Marck et al., 2002). An additional two required tRNAs in both serovars, one shared and one with a differing anticodon, contain Gln anticodons and are part of a polycistronic tRNA operon containing other required tRNAs. This operon is conserved in *E. coli* with the exception of an additional tRNA-Gln at the 3' end that has been lost in the *Salmonella* lineage. It is possible that transposon insertions early in the operon may interfere with processing of the polycistronic transcript in to mature tRNAs. Finally, I did not observe insertions in a tRNA-Met and a tRNA-Val in *S.* Typhi and *S.* Typhimurium, respectively.

Using this analysis of the tRNAs we estimate a worst-case PPV for these short molecules (∼76 bases) at 81%, in line with my previous estimates for conserved protein-coding genes, and a FPR of <4%, higher than for protein-coding genes but still well within the typical tolerance of high-throughput experiments. This assumes that the "required" operonic tRNA-Glns and the serovar-specific tRNA-Met and tRNA-Val are all false positives; it is not clear that this is in fact the case.

Surveying the shared required ncRNA content of both serovars (see table 2.7), I found that the RNA components of the signal recognition particle (SRP) and RNase P, two universally conserved ncRNAs, are required as expected. The SRP is an essential component of the cellular secretion machinery, while RNase P is necessary for the maturation of tRNAs. I also found a number of required known and potential cis-regulatory molecules associated with genes required for growth under laboratory conditions in both serovars. The FMN riboswitch controls *ribB*, a 3,4-dihydroxy-2-butanone 4-phosphate synthase involved in riboflavin biosynthesis, in response to flavin mononucleotide concentrations (Winkler et al., 2002). Additionally, I was able to assign putative functions to a number of previously uncharacterized required non-coding transcripts through their 5′ association with required genes. SroE, a 90 nucleotide molecule discovered in an early sRNA screen (Vogel et al., 2003), is consistently located at the 5′ end of the required *hisS* gene across its phylogenic distribution in the Enterobacteriaceae. Given this consistent association and the function of HisS as a histidyl-tRNA synthetase, I hypothesize that this region may act in a manner similar to a T-box leader, inducing or repressing expression in response

to tRNA-His levels. The *thrU* leader sequence, recently discovered in a deep-sequencing screen of *E. coli* (Raghavan et al., 2011), appears to regulate a polycistronic operon of required singleton wobble tRNAs. Three additional required cis-regulatory elements, t44, S15, and StyR-8, are associated with required ribosomal proteins, highlighting the central role ncRNA elements play in regulating fundamental cellular processes.

Table 2.7: Candidate required ncRNAs greater than 60 nucleotides in length, excluding rRNA and tRNA. Known and putative non-coding elements classified as required or ambiguous in this screen. Required ncRNAs have a log-likelihood ratio (LLR) between required and non-required models of < -2; see Methods. * †, ncRNAs which are amibiguous (LLR between -2 and 2) in S. Typhi(*) or in S. Typhimurium(†). Hfq-binding annotations are taken from Chao et al. (2012). The downstream protein-coding genes columns report annotated CDS or ribosomal RNA start sites within 100 bases of each candidate required non-coding element on either strand, and whether these downstream sequences are also classified as required.

Name	Rfam accession	Function	Hfq-binding	Downstream protein-coding gene(s)	Downstream gene required	References
Required or ambiguous in both *S.* Typhi and *S.* Typhimurium						
SRP	RF00169	RNA component of the signal recognition particle				Rosenblad et al. (2009)
RNase P	RF00010	RNA component of RNase P		*ybaZ*	N	Frank et al. (1998)
RFN	RF00050	FMN-sensing riboswitch controlling the *ribB* gene		*ribB*	Y	Winkler et al. (2002)
SroE	RF00371	Putative cis-regulatory element controlling the *hisS* gene		*hisS*	Y	Vogel et al. (2003)
ThrU Leader	NA	Putative cis-regulatory element controlling the ThrU tRNA operon				Raghavan et al. (2011)
t44	RF00127	Cis-regulatory element controlling the ribosomal *rpsB* gene		*rpsB*	Y	Tjaden et al. (2002); Aseev et al. (2008); Meyer et al. (2009)
S15†	RF00114	Translational regulator of the ribosomal S15 protein		*rpsO*	Y	Benard et al. (1996)
StyR-8	NA	Putative cis-regulatory element controlling the ribosomal *rpmB* gene		*rpmB*	Y	Chinni et al. (2010)
MicA	RF00078	sRNA involved in cellular response to extracytoplasmic stress	Y	*luxS*	N	Vogel (2009b)
DsrA†	RF00014	sRNA regulator of H-NS	Y	*mngB*	N	Lease et al. (1998)
STnc10	NA	Putative sRNA		*nhaA*	N	Sittka et al. (2008)
STnc60†	NA	Putative sRNA				Sittka et al. (2008)
STnc840	NA	Verified sRNA derived from 3' UTR of the *flgL* gene	Y			Chao et al. (2012)
IS0420*†	NA	Putative ncRNA		*rmf*	N	Raghavan et al. (2011); Chen et al. (2002)
RGO0†	NA	Putative sRNA identified in *E. coli*				Raghavan et al. (2011)
Required or ambiguous in *S.* Typhimurium only						
rne5	RF00040	RNase E autoregulatory 5' element		*rne*	Y	Diwa et al. (2000)
RydC	RF00505	sRNA regulator of the *yejABEF* ABC transporter	Y			Antal et al. (2005)
RydB	RF00118	Putative ncRNA				Wassarman et al. (2001)
STnc510	NA	Putative sRNA		*pagD/pagC*	Y/N	Sittka et al. (2008)
STnc460†	NA	Putative sRNA				Sittka et al. (2008)
STnc170	NA	Putative sRNA		SL1458	N	Sittka et al. (2008)
STnc130	NA	Putative sRNA		*dmsA*	N	Sittka et al. (2008)
RseX	RF01401	sRNA regulator of OmpA and OmpC	Y			Douchin et al. (2006)
IsrJ	RF01393	sRNA regulator of SPI-1 effector protein secretion				Sittka et al. (2008); Padalon-Brauch et al. (2008)

IsrI	RF01392	Island-encoded Hfq-binding sRNA	Y	SL1028	Y	Sittka et al. (2008); Padalon-Brauch et al. (2008); Chao et al. (2012)
Required or ambiguous in *S.* Typhi only						
RybB	RF00110	sRNA involved in cellular response to extracytoplasmic stress	Y			Vogel (2009b)
tk5*	NA	Putative ncRNA				Raghavan et al. (2011); Rivas et al. (2001)
STnc750	NA	Verified sRNA	Y	*speB*	N	Kröger et al. (2012); Chao et al. (2012)
StyR-44*	RF01830	Putative multicopy (2/6 copies required in *S.* Typhi) ncRNA associated with ribosomal RNA operon		23S rRNA	N	Chinni et al. (2010)
tp2	NA	Putative ncRNA		*aceE*	N	Raghavan et al. (2011); Rivas et al. (2001)
RdlD	RF01813	RdlD RNA anti-toxin, 1/2 copies required in *S.* Typhi				Kawano et al. (2002)
STnc120*	NA	Putative sRNA				Sittka et al. (2008)
tp28*	NA	Putative ncRNA		*fur*	N	Raghavan et al. (2011); Rivas et al. (2001)
Phe Leader*	RF01859	Phenylalanine peptide leader sequence associated with the required *pheST* operon		*pheS*	Y	Zurawski et al. (1978)
RimP Leader	RF01770	Putative cis-regulator of the *rimP-nusA-infB* operon		*rimP*	Y	Naville et al. (2010)
GlmY	RF00128	Trans-acting regulator of the *glmS* gene				Urban et al. (2008)

2.3.5 sRNAs required for competitive growth

Inferring functions for potential trans-acting ncRNA molecules, such as anti-sense binding small RNAs (sRNAs), from requirement patterns alone is more difficult than for cis-acting elements, as one cannot rely on adjacent genes to provide any information. It is also important to keep in mind that TraDIS assays requirements after a brief competition within a large library of mutants on permissive media. This may be particularly important when surveying the bacterial sRNAs, which are known to participate in responses to stress (Vogel, 2009a).

This is demonstrated by two sRNAs involved in the σ^E-mediated extracytoplamic stress response, RybB and RseX, both of which can be successfully knocked out in *S.* Typhimurium (83). In *S.* Typhi, *rpoE* is required, as it also is in *E. coli* (Baba et al., 2006; De Las Penas et al., 1997). However, in *S.* Typhimurium, *rpoE* tolerates a heavy insertion load, implying that σ^E mutants are not disadvantaged in competitive growth. In *S.* Typhimurium, the sRNA RseX is required. Overexpression of RseX has previously been shown to compensate for σ^E essentiality in *E. coli* by leading to the degradation of *ompA* and *ompC* transcripts (85). This suggests that RseX may also be short-circuiting

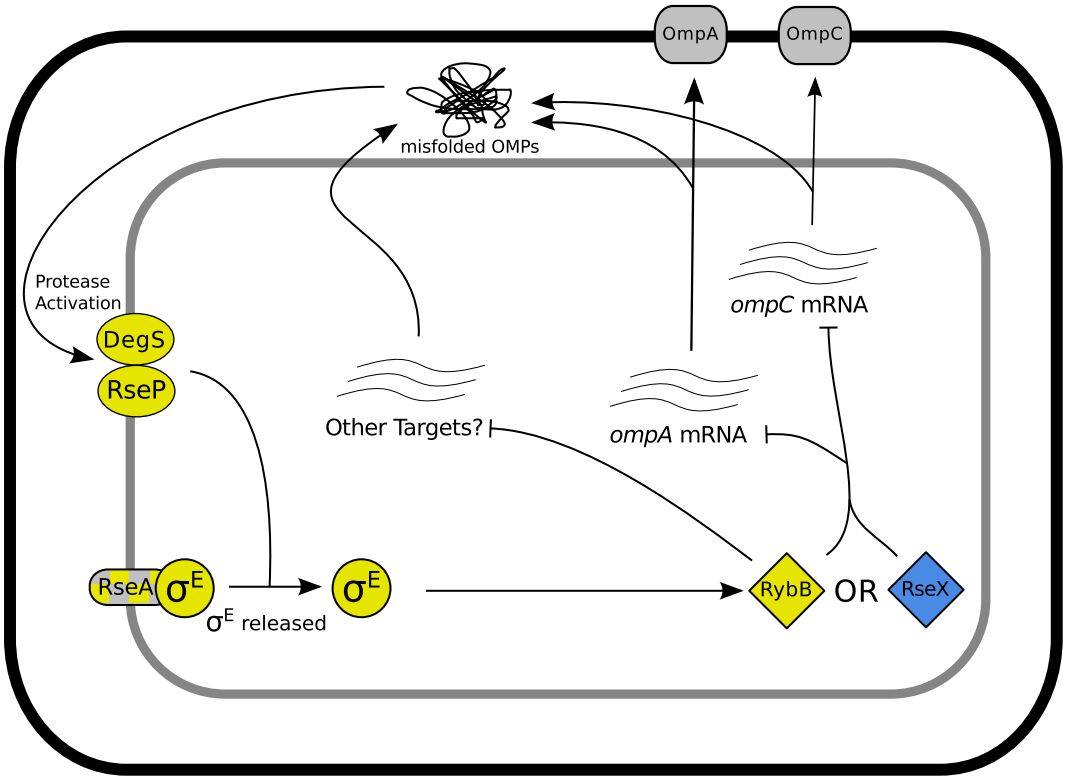


Figure 2.7: Proposed differences in sRNA utilization. Diagram illustrating inferred required sRNA regulatory networks under TraDIS. Molecules required in *S.* Typhi are highlighted in yellow and in *S.* Typhimurium are highlighted in blue. RseA, in yellow/grey check, is ambiguous in *S.* Typhi. Non-required molecules are in grey. Diamonds indicate sRNAs, circles regulatory proteins, ovals proteases, oblong shapes are membrane-anchored proteins, and rounded squares are outer membrane porins.

the σ^E stress response network in *S.* Typhimurium (figure 2.7). To our knowledge, this is the first evidence of a native (i.e. not experimentally induced) activity of RseX.

S. Typhi on the other hand requires σ^E along with its activating proteases RseP and DegS and anchoring protein RseA, as well as the σ^E-dependent sRNA RybB, which also regulates OmpA and OmpC in *S.* Typhimurium, along with a host of other OMPs (Papenfort et al., 2006). It is unclear why the σ^E response is required in *S.* Typhi but not *S.* Typhimurium, though it may partially be due to the major differences in the cell wall and outer membrane between the two serovars. In addition, there are significant differences in the OMP content of the *S.* Typhi and *S.* Typhimurium membranes that

may be driving alternative mechanisms for coping with membrane stress. For instance, *S.* Typhi completely lacks OmpD, a major component of the *S.* Typhimurium outer membrane (Santiviago et al., 2003) and a known target of RybB (Vogel, 2009a).

Two additional sRNAs involved in stress response are also required by both *S.* Typhi and *S.* Typhimurium. The first, MicA, is known to regulate *ompA* and the *lamB* porin-coding gene in *S.* Typhimurium (Bossi et al., 2007), contributing to the extracytoplasmic stress response. The second, DsrA, has been shown to negatively regulate the nucleoid-forming protein H-NS and enhance translation of the stationary-phase alternative sigma factor σ^S in *E. coli* (Lease et al., 1998), though its regulation of σ^S does not appear to be conserved in *S.* Typhimurium (Jones et al., 2006). Both have been previously deleted in *S.* Typhimurium, and so are not essential. H-NS knockouts have previously been shown to have severe growth defects in *S.* Typhimurium that can be rescued by compensatory mutations in either the *phoPQ* two-component system or *rpoS*, implying that the lack of H-NS is allowing normally silenced detrimental regions to be transcribed (Navarre et al., 2006). As MicA has recently been shown to negatively regulate PhoPQ expression in E. coli (Coornaert et al., 2010), it is tempting to speculate that MicA may be moderating the effects of DsrA-induced H-NS repression; however, it is currently unclear whether sRNA regulons are sufficiently conserved between *E. coli* and *S.* enterica to justify this hypothesis.

2.4 Conclusions

The extremely high resolution of TraDIS has allowed the assaying of gene requirements in two very closely related salmonellae with different host ranges. I found, under laboratory conditions, that 58 genes present in both serovars were required in only one, suggesting that identical gene products do not necessarily have the same phenotypic effects in the two different serovar backgrounds. Many of these genes occur in genomic regions or metabolic systems which contain pseudogenes and/or have undergone reorganization since the divergence of *S.* Typhi and *S.* Typhimurium, demonstrating the complementarity of TraDIS and phylogenetic analysis. These changes may in part explain differences observed in the pathogenicity and host specificity of these two serovars. In particular, *S.* Typhimurium showed a requirement for cell surface structure biosynthesis genes; this may be partially explained by the fact that *S.* Typhi expresses the Vi-antigen which

masks the cell surface, though these genes are not required for survival in our assay. *S.* Typhi on the other hand has a requirement for iron uptake through the *fep* system, which enables ferric enterobactin transport. This dependence on enterobactin suggests that *S.* Typhi is highly adapted to the iron-scarce environments it encounters during systemic infections. Furthermore, this appears to represent a single point of failure in the *S.* Typhi iron utilization pathways, and may present an attractive target for narrow-spectrum antibiotics.

Of the approximately 4500 protein coding genes present in each serovar, only about 350 were sufficiently depleted in transposon insertions to be classified as required for growth in rich media. This means that over 92% of the coding genome has sufficient insertion density to be queried in future assays. Dense transposon mutagenesis libraries have been used to assay gene requirements under conditions relevant for infection, including *S.* Typhi survival in bile (Langridge et al., 2009), *Mycobacterium tuberculosis* catabolism of cholesterol (Griffin et al., 2011), drug resistance in *Pseudomonas aeruginosa* (Gallagher et al., 2011), and *Haemophilus influenzae* survival in the lung (Gawronski et al., 2009). I expect that parallel experiments querying gene requirements under the same conditions in both serovars examined in this study will yield further insights in to the differences in the infective process between Typhi and Typhimurium, and ultimately the pathways that underlie host-adaptation.

Both serovars possess substantial complements of horizontally-acquired DNA. I have been able to use TraDIS to assay these recently acquired sequences. In particular, I have been able to identify, on a chromosome wide scale, active prophage through the requirement for their repressors. The P4 phage utilizes an RNA-based system to make decisions regarding cell fate, and structurally similar systems are used by P1, P7, and N15 phage (Citron et al., 1990; Ravin et al., 1999). C4-like transcripts have been regarded as the primary repressor of lytic functions, though the IsrK-like sequence is known to be essential to the establishment of lysogeny in P4 and is transcribed in at least two phage types (Sabbattini et al., 1995; Ravin et al., 1999). These observations in *S.* Typhi suggest an important role for the IsrK-like sequence in maintenance of the lysogenic state in P4-like phage, though the mechanism remains unclear.

Recent advances in high-throughput sequencing have greatly enhanced our ability to detect novel transcripts, such as ncRNAs and short open reading frames (sORFs). In fact, our ability to identify these transcripts now far out-strips our ability to experimentally

characterize these sequences. There have been previous efforts at high-throughput characterization of bacterial sRNAs and sORFs in enteric bacteria; however, these have relied on labor-intensive directed knockout libraries (Santiviago et al., 2009; Hobbs et al., 2010). Here I have demonstrated that TraDIS has sufficient resolution to reliably query genomic regions as short as 60 bases, in agreement with a recent high-throughput transposon mutagenesis study in the α-proteobacteria *Caulobacter crescentus* (Christen et al., 2011). This method has the major advantage that library construction does not rely upon genome annotation, and newly discovered elements can be surveyed with no further laboratory work.

I have been able to assign putative functions to a number of ncRNAs using TraDIS though consideration of their genomic and experimental context. In addition, ncRNA characterization generally is done in model organisms like *E. coli* or *S.* Typhimurium, and it is unclear how stable ncRNA regulatory networks are over evolutionary time. By assaying two serovars of *Salmonella* with the same method under the same conditions, I have seen hints that there may be differences in sRNA regulatory networks between *S.* Typhi and *S.* Typhimurium. In particular, I have found that under the same experimental conditions, *S.* Typhi appears to rely on the σ^E stress response pathway while *S.* Typhimurium does not; it is tempting to speculate that this difference in stress response is mediated by the observed difference in requirement for two sRNAs, RybB and RseX. I believe that this combination of high-throughput transposon mutagenesis with a careful consideration of the systems context of individual genes provides a powerful tool for the generation of functional hypotheses. I anticipate that the construction of TraDIS libraries in additional organisms, as well as the passing of these libraries through relevant experimental conditions, will provide further insights into the function of bacterial ncRNAs in addition to the protein-coding gene complement.

Chapter 3

Methods for the analysis of TraDIS experiments, with an application to *Salmonella* macrophage invasion

Section 3.2 describes a collaborative study with Gemma C. Langridge (Pathogen Genomics, Wellcome Trust Sanger Institute). Gemma performed all laboratory experiments described in this chapter unless otherwise noted.

3.1 Introduction

In the previous chapter, I described the results of a study predicting and comparing the genes required for robust growth of two *Salmonella* serovars in standard laboratory media. While this revealed interesting aspects of *Salmonella* biology, linking these findings to *Salmonella*'s infective niche in the human host is difficult. However, transposon-insertion sequencing can be used to interrogate infective conditions directly (reviewed previously in section 1.5): by comparing libraries passed through a condition of interest to control libraries, we can determine the genomic regions involved in survival in that condition. In this chapter, I describe a pipeline I have devised for the analysis of such experiments, illustrated with an experiment assaying genes required for *S.* Typhi and Typhimurium invasion of (or uptake into) human macrophage. These methods have been adopted by Pathogen Informatics at the Sanger, form the basis of the current Sanger

pipelines for analysis of TraDIS experiments, and are currently being used in a variety of transposon-insertion sequencing studies.

3.1.1 *Salmonella* interactions with macrophage

As previously described in section 2.1, the ability to invade and survive in host cells was a major factor in the early evolution of *S. enterica* subspecies *enterica*; survival in macrophages in particular is known to be necessary for virulence (Fields et al., 1986). This ability appears to have been largely driven by the acquisition of two horizontally-acquired pathogenicity islands, SPI-1 and -2. Due to the availability of a mouse model of systemic infection (Santos et al., 2001), most of what is known about *Salmonella* interactions with host cells is derived from studies of *S.* Typhimurium infection.

S. Typhimurium infections of either epithelial or phagocytic cells appear to follow broadly similar paths (Figueira et al. (2012), see also figure 3.1). On encountering a suitable host cell, the bacterium adheres using an array of fimbrial adhesins (Bäumler et al., 1996; Velden et al., 1998). The SPI-1 T3SS, a needle-like complex spanning the periplasm and presenting its tip to the exterior of the bacterial cell (Mueller et al., 2008), induces membrane ruffling in the host cell through secretion of effector proteins (Zhou et al., 2001), facilitating bacterial uptake. While use of this mechanisms is not strictly necessary for entrance to phagocytic cells such as macrophage, *S.* Typhimurium strains unable to induce ruffling are taken up six to ten times less efficiently than the wild-type (Monack et al., 1996), though the entry mechanism does not ultimately affect cell fate (Rathman et al., 1997).

Once entry has been gained to the cell, through either active invasion or phagocytic engulfment, *S.* Typhimurium begins expressing a second T3SS encoded on SPI-2. The effectors secreted by this T3SS allow *S.* Typhimurium to remodel the *Salmonella* containing vacuole (SCV), and even modulate host immune signalling (see figure 3.1). There is some controversy as to whether or not the SCV undergoes fusion with lysosomes; a recent study suggests it does, but that the activity of these lysosomes is first modulated by the SPI-2 effector SifA (McGourty et al., 2012). Little is known about the growth conditions *S.* Typhimurium faces within the SCV, though transcriptomic studies suggest it is aerobic, mildly acidic, rich in gluconate, and limited in aromatic amino acids, purines, and pyrimidines (Eriksson et al., 2003; Hautefort et al., 2008).

Our understanding of how these findings relate to *S.* Typhi infections of human macrophage is limited, largely due to the lack of a non-human model organism for infection by this serovar. A recent study suggests that SPI-2 may not even be necessary for *S.* Typhi invasion of and survival in human macrophages (Forest et al., 2010), though SPI-2 genes are known to be expressed by *S.* Typhi in macrophages (Faucher et al., 2006) and a SPI-2 deletion mutant was previously shown to be attenuated under these conditions (Khan et al., 2003). Regardless, it is well established that the genotype of both the *Salmonella* strain used and the macrophage can have profound effects on the course of infection. A number of studies comparing a variety of *Salmonella* serovars infecting murine-, human-, and even chicken-derived macrophages have repeatedly shown that serovars exhibit remarkably different behaviors under the same conditions (Buchmeier et al., 1989; Vladoianu et al., 1990; Schwan et al., 2000; Okamura et al., 2005); these differences appear to correlate somewhat with the degree of host-adapation exhibited by the serovar. In this study we compare our *Salmonella* TraDIS libraries following uptake by human macrophage in the hopes of uncovering genomic factors underlying these differences in behavior.

3.1.2 Conditional gene fitness

Determining conditional gene fitness presents a somewhat different problem to that addressed in the previous chapter, predicting and comparing "essential" genes under the conditions of library creation. In predicting gene essentiality, we had a single time point representing the initial growth of the library on rich media, while in identifying conditional gene fitness (measured as the relative expansion or contraction of mutant populations) we are always comparing changes in mutant fitness with respect to fitness in a baseline condition. The ratio of reads between the two conditions is taken as indicative of differences in relative mutant prevalences between them. In some ways, this makes the problem of identifying genes with strong fitness effects easier: as we are primarily interested in the ratio of various insertion mutants present between the two conditions, effects that may confound the prediction of simple gene essentiality are effectively "zeroed out". More explicitly, whether low insertion density in the initial library occurs due to chance, nucleotide composition bias, or the exclusionary effects of high-density DNA-binding proteins (described in section 2.3.3) does not matter – these

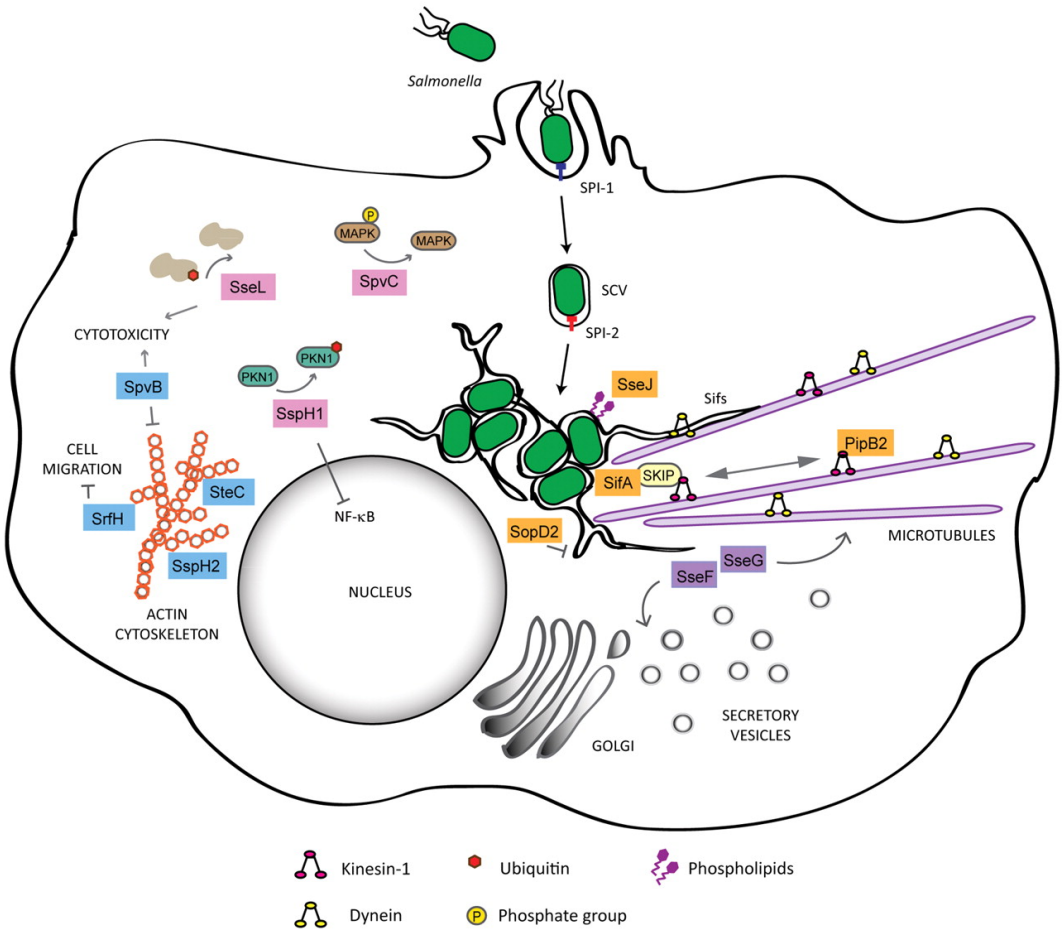


Figure 3.1: Biogenesis of the *Samonella* containing vacuole (SCV). *Salmonella* adheres to the outer membrane of host cells, and uses the SPI-1 T3SS and its associated effectors to induce membrane ruffling and entry into the SCV. The SPI-2 T3SS functions mainly in maintenance of the SCV, through the action of the effectors SifA, SopD2, SseJ and PipB2 (orange boxes), and its localization near the Golgi of host cells, mediated by SseF and SseG (purple boxes). Other effectors are involved in modulation of host immune signalling (SpvC, SspH1 and SseL; pink boxes) or target the host cytoskeleton (SteC, SpvB, SspH2 and SrfH; blue boxes). Reproduced from Figueira et al. (2012) under a Creative Commons Attribution License (CCAL).

regions can simply be identified as not producing sufficient reads over insertion-sites to be assayed and removed from the analysis.

In many ways, the problem of investigating the statistical and biological significance of ratios of reads over insertion sites resembles established analyses developed for differential RNA-seq analysis. In the following sections I describe the application of these methods to the problem of determining conditional gene fitness using the *Salmonella* macrophage infection dataset as an example.

3.2 Experimental methods

Gemma C. Langridge performed all laboratory experiments described in this chapter, as well as read mapping; condensed descriptions are included here for completeness. Silvia Pinero prepared the THP-1 cells for infection. Sabine Eckert and Daniel Turner (Wellcome Trust Sanger Institute) performed the nucleotide sequencing. A more detailed description of the experimental methods is available in Langridge (2010), including preliminary assessments of bacterial strain ability to grow in RPMI, invade THP-1 derived macrophage, and experiment optimization.

3.2.1 Strains and cell lines

These experiments were performed with *S.* Typhi WT174 and *S.* Typhimurium SL3261 transposon mutant libraries, described in chapter 3. Annotations and orthology predictions used are as in chapter 3. Human monocytic cell line THP-1 was used for cell infections.

3.2.2 Preparation of THP-1 cells

THP-1 cells were grown up from frozen stocks in RPMI-1640 supplemented with 10% heat-inactivated foetal bovine serum and 2 mM L-glutamine, and incubated without shaking in vented flasks (VWR, Lutterworth, UK) at 37°C in the presence of 5% CO_2. Culture volumes were split and given fresh media every 3-4 days until the desired volume and cell density was achieved. Phorbol myristate acetate (PMA) was used to differentiate the THP-1 monocytes. Briefly, approximately 212 cells in 4 mL supplemented RPMI

containing 0.125 ng/mL PMA were seeded into each well of a 6-well plate and incubated for six days at 37°C in 5% CO_2. On the day of infection, the PMA-containing media was removed, cells were washed with dPBS and fresh warmed, supplemented RPMI was added to maintain the cells while the bacterial inoculum was prepared.

3.2.3 Preparation of transposon libraries

Frozen stocks of the Typhi library were found to be at half the concentration of the Typhimurium library by OD600. To ensure similar concentrations for the infection assay, a 1 in 5000 dilution of the Typhi library and a 1 in 10,000 dilution of the Typhimurium library was used to inoculate the growth medium. Cultures of each transposon library were grown with shaking in 100 mL of RPMI-1640 supplemented with 0.3 g/L L-glutamine and buffered with 10 mL 1 M MOPS at 37°C for 16 hours. These cultures were sub-cultured 1 in 20 into fresh RPMI supplemented and buffered as before, and grown for between 3 and 4 hours to mid-log phase (OD600 of 2.4).

3.2.4 Infection assay

Five 6-well plates were used for each run of the assay. In total, 29 wells were infected with the bacterial inoculum and one served as a blank control for eukaryotic cell contamination. At the start of the assay, media was removed from all wells except for the blank control, and a 3 mL bacterial inoculum was added to each experimental well. The plates were centrifuged for 5 minutes at 600 x g and incubated at 37°C in 5% CO_2 for 30 minutes. A 4-6 mL aliquot of the inoculum was processed for genomic DNA as the TraDIS control. After 30 minutes, media was removed from all wells, and fresh RPMI additionally supplemented with 100 μg/mL gentamicin was added. After 2 hours the wells were washed 3 times in plain dPBS. Following washing, 500 μL of 1% Triton-X-100 was added to each well to lyse the eukaryotic cells, mixed well by pipetting, and incubated at 37°C in 5% CO_2 for 2 minutes. The cell suspensions from all experimental wells were pooled for bacterial DNA extraction. Genomic DNA was extracted using the Qiagen DNeasy Blood and Tissue kit, according to the manufacturers protocol for Gram negative bacteria. Sequencing was performed as described in section 2.2.4.

3.3 Analysis of conditional gene fitness using TraDIS

3.3.1 Experimental design

The goal for this experiment was to determine the differences in gene requirements for human macrophage infection in two *Salmonella* serovars: Typhimurium, a host-generalist, and Typhi, host-restricted to humans as described in the previous chapter. To this end, infection assays of THP-1 monocytes were performed in triplicate with transposon libraries for each serovar at high multiplicities of infection in an attempt to avoid bottleneck effects. These were compared to libraries grown in cell culture medium (RPMI), to control for any incidental changes in library composition due to growth in this medium.

3.3.2 Mapping insertion sites

Read mapping is a special case of one of the oldest problems in bioinformatics, aligning a short sequence of length n to a much longer sequence, or database of sequences, of length m. An optimal solution (with respect to a particular sequence similarity scoring scheme) for this problem using dynamic programming was first proposed by Smith et al. (1981), building on previous work by Needleman et al. (1970). Unfortunately, this method requires construction of a dynamic programming matrix of size $n \times m$, which quickly becomes impractical for large m due to both time and memory constraints. Heuristic solutions to this problem have been developed, starting with the FASTA and BLAST algorithms (Lipman et al., 1985; Altschul et al., 1990). The basic idea behind these heuristics is to rapidly search for identical matches using a hash of the sequence database before performing a full Smith-Waterman style local alignment around this match. For the case of mapping reads to larger eukaryotic genomes, more powerful heuristics, such as the Burrows-Wheeler transform (Burrows et al., 1994; Langmead et al., 2009; Li et al., 2010), may be required due to time and space constraints. However as we are working with relatively small bacterial genomes, MAQ (Li et al., 2008) has been used here, which is similar in spirit to FASTA or BLAST, but with additional refinements to deal with repetitive genomic regions and to assessing alignment quality.

Table 3.1: Summary statistics for macrophage infection assay sequencing runs. Table columns as follows: 1, description; 2, total sequencing reads; 3, reads containing transposon tag; 4, reads mapped to chromosome with quality score greater than 20; 5, number of insertions recovered. STY: *S.* Typhi; STM: *S.* Typhimurium.

Description	Reads	Reads tagged	Reads mapped	Insertion sites
STY control 1	11107014	10534361	9722100	154356
STY control 2	10983030	10016035	8868829	193417
STY control 3	13506872	12168442	11062549	180998
STY infection 1	7526390	4193529	2304138	90218
STY infection 2	8630360	4166256	2000771	73154
STY infection 3	8215834	4323817	2459573	98894
STM control 1	14583559	14314003	9318191	365266
STM control 2	18119496	17494267	11458349	464036
STM control 3	13565707	12457266	7312946	179702
STM infection 1	3292265	2972803	2033041	41775
STM infection 2	6444469	5351193	3732480	59476
STM infection 3	13012186	12124834	9633788	43110

3.3.3 Quality control

We can asses the quality of TraDIS experiments on multiple levels: the number of reads containing transposon tags and mapping to the genome, the number of insertion sites recovered, the correlation between the numbers of reads recovered for each gene in replicated experiments, and clustering experiments using a dimensionality reduction technique such as principal component analysis (PCA).

Summary statistics of the sequencing runs for this study are presented in table 3.1. Total read yield varied from ∼3.3 to ∼18.1 million reads, with lower yields generally observed for the infection libraries. Similarly, the percentage of reads containing exact matches to transposon sequence is significantly lower in the *S.* Typhi infection samples, which may be a result of low read quality obscuring the sequence. However, despite these issues, over two million reads over insertion sites were recovered in every sample which provides adequate coverage for this assay. Interestingly, the number of unique insertion sites recovered from the *S.* Typhimurium infection assays was approximately half that observed in the *S.* Typhi assay in every replicate, despite having an apparently more complex inoculum. This is suggestive of either stronger selective pressure, a more severe bottleneck effect, or both for *S.* Typhimurium compared to *S.* Typhi during infection of human macrophage, as might be expected given the latter's host adaptation.

Linear correlation coefficients, reported in table 3.2 lend some credence to this idea that *S.* Typhimurium may be experiencing a more severe bottleneck leading to the incidental loss of mutants during infection, possibly due to the killing effects of the

Table 3.2: Pearson's r between replicated TraDIS experiments. Correlations of reads over genic and non-coding RNA features between replicated control and infection assays, rounded down to nearest hundredth. Y: *S.* Typhi; M: *S.* Typhimurium; C: Control; I: Infection.

	Y C1	**Y C2**	**Y C3**	**Y I1**	**Y I2**	**Y I3**	**M C1**	**M C2**	**M C3**	**M I1**	**M I2**	**M I3**
Y C1	1.00	0.99	0.99	0.65	0.69	0.72	0.43	0.43	0.48	0.34	0.39	0.43
Y C2	0.99	1.00	0.99	0.65	0.70	0.72	0.42	0.43	0.48	0.33	0.39	0.43
Y C3	0.99	0.99	1.00	0.67	0.71	0.74	0.44	0.44	0.49	0.34	0.40	0.45
Y I1	0.65	0.65	0.67	1.00	0.99	0.99	0.26	0.28	0.32	0.30	0.31	0.49
Y I2	0.69	0.70	0.71	0.99	1.00	0.99	0.28	0.28	0.34	0.31	0.33	0.49
Y I3	0.72	0.72	0.74	0.99	0.99	1.00	0.29	0.29	0.35	0.31	0.33	0.50
M C1	0.43	0.42	0.44	0.26	0.28	0.29	1.00	0.99	0.93	0.74	0.85	0.76
M C2	0.43	0.43	0.44	0.26	0.28	0.29	0.99	1.00	0.93	0.73	0.85	0.77
M C3	0.48	0.48	0.49	0.32	0.34	0.35	0.93	0.93	1.00	0.69	0.80	0.75
M I1	0.34	0.33	0.34	0.30	0.31	0.31	0.74	0.73	0.69	1.00	0.74	0.68
M I2	0.39	0.39	0.40	0.31	0.33	0.31	0.85	0.85	0.80	0.74	1.00	0.72
M I3	0.43	0.43	0.45	0.49	0.49	0.50	0.76	0.75	0.75	0.68	0.72	1.00

macrophage. Correlations between replicate experiments are over .99 with two notable exceptions. The first is in the third replicate of the *S.* Typhimurium assay. Due to failure of this replicate during the current study, an earlier 2 hour time point from optimization experiments (Langridge, 2010) was used, so the lower correlation between the third control replicate and replicates 1 and 2 may be explained by this sample being handled at a different time and sequenced earlier on a different machine. However, the correlation coefficient between the third control replicate and replicates 1 and 2 is still well over .9, indicating that it still largely agrees with the later experiments.

The other discrepancy is in the correlation between *S.* Typhimurium infection experiments, with coefficients ranging between .68 and .74. This is still a high positive correlation; however it does not reach the level observed in the other replicated experiments in this study. This is again suggestive of a bottleneck effect in this assay. If the loss of particular mutants were purely due to selection, we would expect a high correlation, as these losses would presumably be reproducible under the same experimental conditions. Rather, it appears that there is some stochasticity in the loss of mutants in this particular experiment, suggesting losses that are incidental to the actual factors underlying infection of human macrophage. As mentioned previously, this may be due to a higher rate of macrophage killing of the non-host adapted *S.* Typhimurium strain used in this study. It has been observed previously that even in *S.* Typhimurium strains capable of successfully infecting macrophage, some proportion of the invading bacteria do not manage to establish a protective SCV (Monack et al., 1996) for reasons that remain unclear. A higher rate of failure in establishing the SCV in human macrophage for *S.* Typhimurium than *S.* Typhi, or even the use of an entirely different mechanism

for survival in macrophage by *S.* Typhi, may explain this difference.

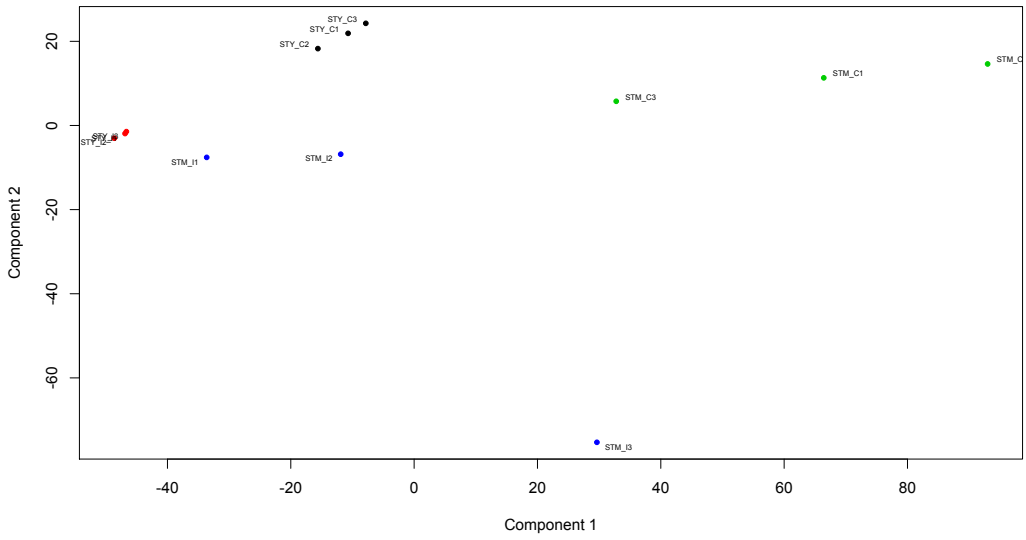


Figure 3.2: Principal component analysis of TraDIS macrophage infection assays. Plot showing samples along first two components of a PCA, representing 55% and 18% of the total variance in the data set, respectively. Replicates appear to cluster together, with the exception of the third Typhimurium infection replicate, which was excluded from the analysis. STY: *S.* Typhi; STM: *S.* Typhimurium; C: control; I: infection.

To further investigate the potential bottleneck effect in *S.* Typhimurium, I performed a principal component analysis (PCA) on all samples. PCA is a mathematical technique for dimensional reduction which identifies linear vectors (components) in a high-dimensional dataset which capture maximal amounts of the variance between samples. This high dimensional data can then be visualized in a lower (e.g. 2 or 3) dimensional space by plotting samples against these components. Samples were centered and scaled to correct for the differences in read counts between experiments. Plots of all samples in this study on the first two principal components, accounting for 55% and 18% of the total variance respectively, are shown in figure 3.2. With the exception of the third *S.* Typhimurium infection experiment, all samples collected under the same conditions cluster on this plot, as would be expected if these results are reporting the effects of differential selection. All infection samples lie to the left of their respective control

samples on the first component, suggesting that the dominant signal in this data is due to the effects of selection during macrophage infection. The fact that two of the three *S.* Typhimurium infection experiments cluster together suggests that this signal is stronger than any stochastic bottleneck effect despite the lower correlations observed between these libraries, and that it should be possible to derive useful information about the conditions faced by *S.* Typhimurium during infection from these experiments. Unfortunately, the third *S.* Typhimurium infection replicate, which was performed separately as described earlier, does not cluster well with these. I performed a similar analysis using the plotMDS function of edgeR (Robinson et al., 2010a), which performs multidimensional scaling using a variance-stabilized distance measure between samples, and came to a similar result. It is unclear why this replicate is so different, and it may be due to differences in experimental set up or sequencing. I excluded this replicate from further analysis on this basis.

3.3.4 Inter-library normalization

Normalization is a critical part of any high-throughput sequencing experiment. As observed in the previous section, even the same experiment repeated on the same machine can lead to very different read counts. The naive approach to solving this problem is simply to scale each sequencing library by some factor so that the total read counts are equivalent. This may be adequate for analysis of technical replicates where gene expression levels are identical between all samples. However, Robinson et al. (2010b) illustrate why this may not be the case for the comparison of sequencing libraries sampling populations under different conditions with a simple thought experiment:

> Imagine we have a sequencing experiment comparing two RNA populations, A and B. In this hypothetical scenario, suppose every gene that is expressed in B is expressed in A with the same number of transcripts. However, assume that sample A also contains a set of genes equal in number and expression that are not expressed in B. Thus, sample A has twice as many total expressed genes as sample B, that is, its RNA production is twice the size of sample B. Suppose that each sample is then sequenced to the same depth. Without any additional adjustment, a gene expressed in both samples will have, on average, half the number of reads from sample A, since the reads are spread

> over twice as many genes. Therefore, the correct normalization would adjust sample A by a factor of 2. (Robinson et al., 2010b)

More generally, we can think of each gene in a sequencing library as representing a slice of a pie. If a particular gene increases in expression (or mutant prevalence for transposon-insertion sequencing such as ours), then the space left in this pie for other genes necessarily shrinks. A scaling normalization which does not take this fact in to account, but simply assumes all pies are the same size would necessarily underestimate expression (or prevalence) for the majority of genes which don't change, while overestimating it for the few that do. A recent study has shown that normalization methods which explicitly account for this problem perform better on both real and simulated data (Dillies et al., 2012). Here I have used the trimmed mean of M-values (TMM) method, which assumes the majority of genomic features do not change in actual expression (or mutant prevalence here) and attempts to align the read counts of these features to produce an appropriate scaling factor (Robinson et al., 2010b).

3.3.5 Identifying fitness effects

3.3.5.1 Theory

Once sequencing libraries have been normalized, the next step in determining fitness effects is the choice of a proper test to determine the significance of changes in read counts. In the previous chapter, I used two test statistics. The first was to test for gene requirements within a particular library, and this was accomplished by fitting gamma distributions to the two modes observed in the empirical distributions of insertion indexes, then setting a threshold based on a log-odds ratio (see figure 2.2). The second was to additionally test for significant differences in read depth between the *S.* Typhimurium and *S.* Typhi libraries. In this case the $\log_2$ read ratios between genomic features in the two libraries were roughly normally distributed, and I was able to set a significance threshold based on a fitted normal curve.

Neither of these tests are entirely appropriate for the present situation of identifying reproducible changes in mutant prevalence in replicated experiments. Most obviously, neither of these test can easily be modified to accommodate replicates, which is essential for robust identification of changes in mutant prevalence. Secondly, both tests are

dependent on manual fitting of gamma or normal distributions, which can not easily or robustly be automated. Standard statistical tests, such as the two sample Student's T-test or Mann-Whitney U-test are not applicable due to the small numbers of replicates (3 here, often 2) because of high experimental overhead in replication. Fortunately, these problems have largely been addressed in modern RNA-seq differential expression analysis software.

The two leading packages for analysis of RNA-seq based differential expression analysis are DESeq (Anders et al., 2010) and edgeR (Robinson et al., 2010a). Both assume that sequence count data is negative binomially distributed. The negative binomial distribution arises naturally in the case of a Poisson process sampling from gamma-distributed random variables (Fisher, 1941). Sequencing of mixed populations of oligonucleotides has long been theorized to behave as a Poisson process, and this has shown to roughly be the case for technical replicates of Illumina RNA-seq runs (Marioni et al., 2008), i.e. repeated sequencing of the same input sample. Other studies have shown that biological RNA-seq and SAGE replicates, i.e. repeated experiments, generate extra-Poisson variability (Lu et al., 2005; Robinson et al., 2007), possibly due to variability in the concentration of the transcripts being sampled, which can be captured by the negative binomial.

This leads naturally to the question, is transposon-insertion sequencing data negative binomially distributed? Obviously, technical replicates of TraDIS experiments will be roughly Poisson distributed, as this is identical to the case of technical replication of RNA-seq. The question then becomes whether the underlying distribution of mutant prevalences being sampled by sequencing can be effectively modelled by a gamma distribution. Theoretical considerations indicate that this model may be appropriate: as subcultures of the mutant library expand, the number of insertion mutants per gene will be the summed result of independent exponentially-expanding clones, which will be gamma distributed assuming the starting populations are roughly equal. The only way to answer this question definitively would be to repeat the same experiment a large number of times, which is impractical. However, this is not necessary. Lu et al. (2005) showed that the negative binomial assumption is highly robust to the actual distribution of the data being assessed. In fact, it appears that the underlying transcript prevalences being sampled by RNA-seq experiments may actually be distributed according to a sum of log-normal distributions (Bengtsson et al., 2005); this does not prevent DESeq and edgeR from performing competitively in benchmarks of differential expression

analysis (Kvam et al., 2012; Soneson et al., 2013). These approaches have previously been successfully applied to other Illumina sequencing-based experiments which likely have different underlying distributions than transcriptomic data, for instance differential analysis of ChIP-seq data (Robinson et al., 2012).

I have used edgeR (Robinson et al., 2010a) for significance testing here, an R package which implements the TMM normalization (Robinson et al., 2010b), an approximation to an empirical Bayes estimation of feature-wise negative binomial dispersion parameters (Robinson et al., 2007), and a version of Fisher's exact test modified to deal with overdispersed data (Robinson et al., 2008) as well as a likelihood-ratio test in the case of multifactorial designs (McCarthy et al., 2012; Lund et al., 2012). After testing, we are interested primarily in two values: the P-value given by the statistical testing which tells us how confident we can be that mutant prevalence differs between two conditions given the estimated negative binomial distribution distribution of read counts, and the $\log_2$ fold-change (logFC) which gives an estimation of the magnitude of the difference. LogFC is calculated as

$$logFC_g = \log(n_{g,b}) - \log(n_{g,a})$$

where the index g indicates the genomic feature being tested, $n_{g,b}$ is the normalized average read count in the test condition, and $n_{g,a}$ is the normalized average read count in the control condition. This subtraction is equivalent to taking the log of the ratio $\frac{n_{g,b}}{n_{g,a}}$, and hence $logFC_g$ becomes unstable for small changes in $n_{g,b}$ as $n_{g,a} \to 0$, and is ultimately undefined when $n_{g,a} = 0$. In the previous chapter I corrected for this by adding a pseudocount to each gene's read count. I take the same approach here, as implemented in edgeR, only since each library has been normalized by a different factor, I rather use the transformation

$$n^T_{g,x} = \log(\frac{n_{g,x}}{L_x} + \frac{2}{\overline{L}})$$

where L is the library size. This has the effect of shrinking unreliable logFCs for features with small read counts, and removing the problem of undefined logFCs.

3.3.5.2 Application to macrophage infection data

Returning to the macrophage infection assays, I first eliminated genomic features from consideration which did not have at least 20 counts per million normalized reads (CPM) in at least three assay or control replicates. This cut-off is arbitrary, but serves the purpose of removing features from consideration which do not have adequate read coverage to deliver biologically significant results in at least one condition. This provides two advantages: firstly, it increases statistical power by reducing the number of simultaneous hypothesis tests that need to be corrected for, and secondly, it eliminates features which may have statistically significant logFCs but may not have large enough mutant populations to determine if these effects are biologically relevant. This reduces the number of genomic features tested from 3882 (including all orthologous coding sequences and non-coding RNAs) to 3596.

I then set up three statistical analyses within the generalized linear model (GLM) framework provided by edgeR, which allows for multi-factorial analyses. The first tests whether the logFC between *S.* Typhimurium infection and control is different from the logFC between *S.* Typhi infection and control. This allows me to discriminate between mutant populations which behave similarly during macrophage invasion in the two serovars (no or small difference in logFCs), from those which behave differently (large difference in logFCs). Of course, this does not allow me to discriminate between mutant populations which are expanding, those that are shrinking, or those which are static in both serovars during invasion - this test only tells if their behavior is similar. Similarly, using this test I can not discriminate between features with differences in logFCs that are the result of mutant expansion in one serovar, or contraction in another. For this reason I performed two additional analyses, testing the significance of logFCs between infection and control in each serovar independently. All p-values have been corrected for multiple testing using the method of Benjamini et al. (1995), controlling for a false discovery rate (FDR) of 10%.

The results of the comparison between *S.* Typhimurium and *S.* Typhi changes in logFC over macrophage infection are shown in figure 3.3, and the individual changes in mutant prevalences for each serovar are shown in figures 3.4 and 3.5. On first viewing these figures, there is a striking difference in the behavior of the *S.* Typhimurium and *S.* Typhi mutant libraries: while *S.* Typhimurium displays a wide spread of changes in

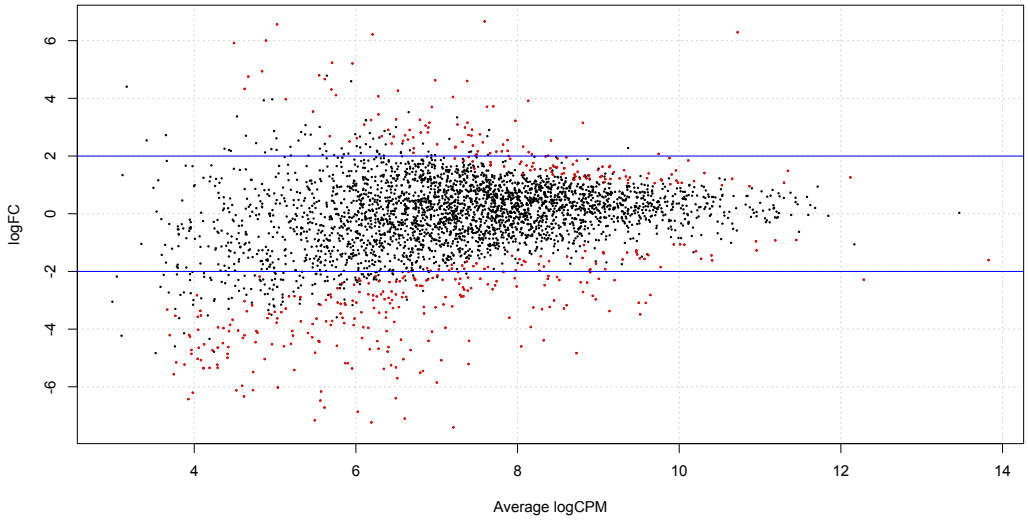


Figure 3.3: Smear plot of differences in logFC over macrophage infection between *S.* Typhimurium and *S.* Typhi. Each point in this plot represents a tested genomic feature. LogFC is reported on the Y-axis, logCPM on the X-axis; statistically significant features at a FDR of 0.1 are in red. The blue lines represent logFCs of |2|, translating to a four-fold difference in logFC in mutant prevalences between the two serovars. Negative values indicate that the *S.* Typhimurium mutant population has shrunk relative to the *S.* Typhi mutant population, and vice versa.

mutant prevalence, 938 of them statistically significant (see Appendix A), indicating a strong selective pressure operating on the library, the composition of the *S.* Typhi library appears nearly unchanged after infection, with only 28 features showing a statistically significant change in mutant prevalence (see table 3.5). In fact it appears that nearly all of the statistically significant differences in logFC between the two libraries over macrophage infection are due to changes in mutant prevalences in the *S.* Typhimurium library. This seems to indicate, on a gross level, that *S.* Typhi is somehow avoiding the brunt of the gauntlet imposed on *S.* Typhimurium in the first two hours of macrophage infection. This may partially be due to the presence of the Vi capsule on *S.* Typhi, which has previously been shown to enhance survival in THP-1 derived macrophage (Hirose et al., 1997) through the creation of a 'stealth' phenotype which reduces the expression of inflammatory factors, such as TNF-α, by the macrophage.

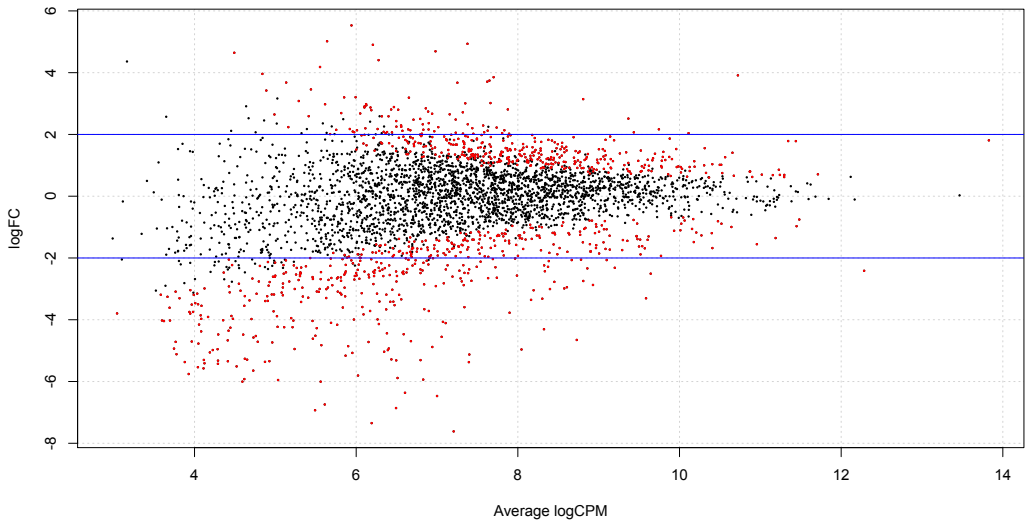


Figure 3.4: Smear plot of logFC in mutant prevalences over macrophage infection in *S.* Typhimurium. Each point in this plot represents a tested genomic feature. LogFC is reported on the Y-axis, logCPM on the X-axis; statistically significant features at a FDR of 0.1 are in red. The blue lines represent logFCs of |2|, translating to a four-fold change in mutant prevalences between infection and control. Negative values indicate a reduction over infection in mutant prevalence, positive values an increase.

3.3.6 Functional analysis of gene sets that affect fitness

Now that I have determined the changes in mutant prevalences in each library over macrophage infection, I am left with the task of determining the biological context and importance of these changes. The traditional approach, taken in the previous chapter with regards to genomic features required for survival under standard laboratory conditions, would be to create a ranked list and work through these features one at a time, researching what is known about them and building a picture of their contribution to survival in the macrophage. This has some distinct advantages, as it allows the investigator to piece together new hypotheses as to gene function from the existing literature. However, while it was possible with <100 genomic features identified as significantly affecting growth in a single serovar, the task becomes extremely time consuming when faced with the ∼1000 genes potentially involved in macrophage infection in *S.* Typhimurium. Hence an alternative, automated approach is required, at least for a first scan of the data.

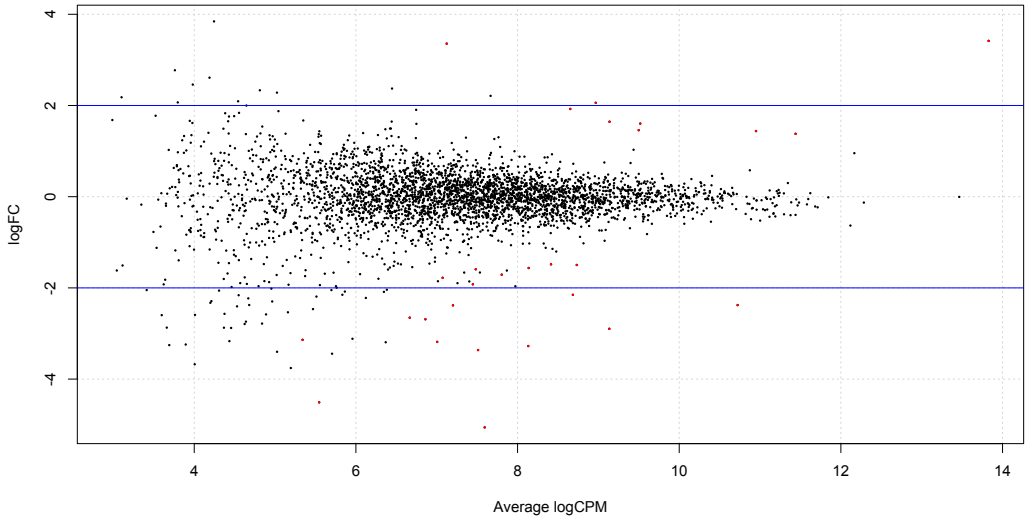


Figure 3.5: Smear plot of logFC in mutant prevalences over macrophage infection in *S.* Typhi. Each point in this plot represents a tested genomic feature. LogFC is reported on the Y-axis, logCPM on the X-axis; statistically significant features at a FDR of 0.1 are in red. The blue lines represent logFCs of |2|, translating to a four-fold change in mutant prevalences between infection and control. Negative values indicate a reduction over infection in mutant prevalence, positive values an increase.

A number of resources exist which could provide a basis for this sort of automated functional analysis of high-throughput experimental data. These include the Gene Ontology (GO) (Gene Ontology Consortium, 2013), MetaCyc (Caspi et al., 2012), TIGRFAM and Genome Properties (Haft et al., 2013), and the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa et al., 2012). Each of these databases has different goals in its curation, and their own unique advantages and disadvantages. For instance, TIGRFAM provides hidden Markov models (HMMs) with attached pathway information, which can be used to annotate pathways and subsystems present in a genome in the absence of annotation. MetaCyc provides similar resources, including tools for filling 'hole' in pathways and subsystems annotated in a genome, based on large manually curated pathway databases covering much of the diversity of life. However, here I have chosen to use the KEGG database as the basis for my analysis, as it is relatively comprehensive, contains annotations for both of the serovars being studied here, and has

a readily available R interface.

Many techniques have been developed for purposes of pathway analysis (Khatri et al., 2012), however, few of them have readily available implementations and many of those that do are tailored to Eukaryotic data. So instead of using a previously developed method, I implemented what I have previously described as a 'walking hypergeometric test' (Croucher et al., 2012) in the context of determining the effect of sequence identity on recombination in *Streptococcus pneumoniae*. In a standard hypergeometric test, one labels genes as being members of a category (in this case a pathway or subsystem), then asks if a random draw of the same size as the significant gene set were taken without replacement, whether one would expect to draw this many (or more) labelled genes by chance. The walking hypergeometric test extends this by walking down an ordered list, in this case sorted by logFC, and performing a hypergeometric test for category enrichment at each entry. This technique is inspired by the test used in the Sylamer microRNA target prediction tool (Dongen et al., 2008), itself inspired by GSEA (Subramanian et al., 2005). An illustration of this test can be seen in figure 3.6.

This method has a number of important advantages over traditional gene enrichment testing. Normally, one would first choose significance cut-offs based on a p-value and logFC, then perform a hypergeometric test using the resulting set as the draw. This can fail to detect enriched categories if the size of the draw is large, as in the case of *S.* Typhimurium here. Additionally, these cut-off are by their nature somewhat arbitrary, and it is possible that a large number of genes with individually (statistically) non-significant effects could be representative of a (biologically) significant effect on an entire pathway or subsystem. Finally, this method also provides an intuitive graphical representation of the test statistic, which allows the viewer to understand the distribution of gene categories in the data.

3.4 Results and Discussion

I applied the walking hypergeometric test to *S.* Typhimurium in order to discover pathways and subsystems involved in the infective process of this organism. Six pathways were found to be significantly enriched in genes with mutant populations undergoing either expansion or contraction during macrophage infection, summarized in table 3.3. The pathways with significantly expanding mutant populations were LPS biosynthesis

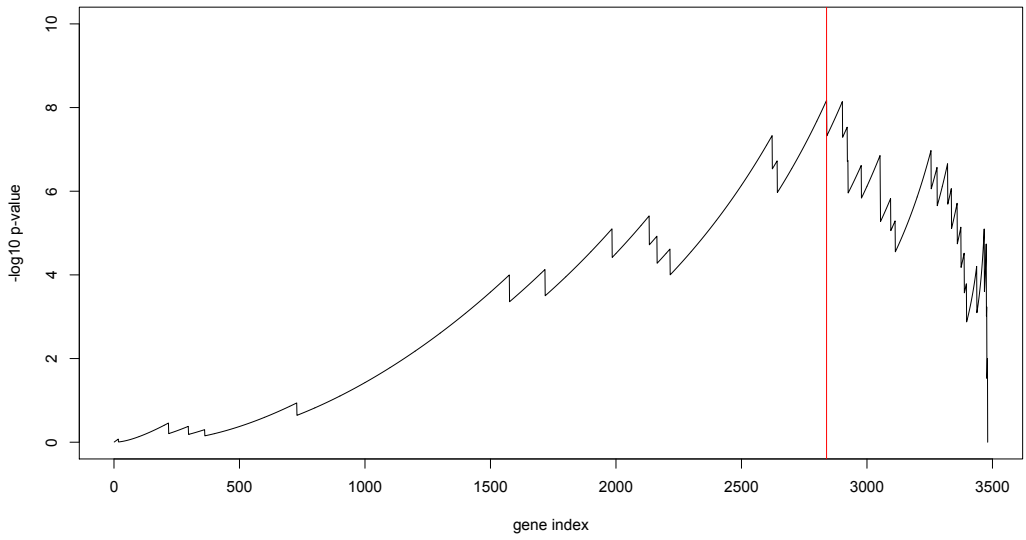


Figure 3.6: Walking hypergeometric test for depletion of insertions in the *S.* Typhimurium flagellar subsystem. The x-axis shows the index of genes sorted on logFC from highest (enrichment in insertions over the experiment) on the left to lowest (depleted in insertions over the experiment). The y-axis shows the $-\log_{10}$ p-value derived from a hypergeometric test at each gene for a higher than expected number of genes in the flagellar subsystem to the right of that position. The red line at index 2839 indicates the position of the minimum p-value of $\sim 6.7 \times 10^{-9}$.

and purine metabolism. LPSs are well known to be antigenic, and in fact are commonly used to activate macrophages for infection assays in the laboratory. It seems likely that mutants defective in LPS biosynthesis are able to survive better due to a reduction in the inflammatory response provoked in the host cell. The SCV is known to be limited in purines (Eriksson et al., 2003; Hautefort et al., 2008), so mutants which do not waste resources synthesizing genes involved in purine metabolism would also have a selective advantage.

Flagellar assembly, bacterial secretion systems, and RNA degradation on the other hand were all found to be enriched in genes with contracting mutant populations. Flagellar assembly is particularly striking (figure 3.7), with 28 of 34 genes in the subsystem exhibit negative logFCs over macrophage invasion. Interestingly, three genes in this subsystem exhibited statistically significant positive fold changes. Most strongly among these

Table 3.3: *S.* Typhimurium pathways putatively involved in macrophage infection. Pathways and subsystems with a walking hypergeometric minimum p-value less than 1×10^{-3}. Table columns as follows: 1, pathway description; 2, identified as being relatively enriched or depleted in mutants; 3, minimum p-value from walking hypergeometric test; 4, number of genes in pathway significant enriched/depleted in mutants; 5, number of genes in pathway with significantly different logFCs compared to *S.* Typhi.

KEGG Pathway	Enriched/Depleted	P-value	Genes	Different from Typhi
Lipopolysaccharide Biosynthesis	Enriched	6.67×10^{-6}	6	9
Purine Metabolism	Enriched	9.51×10^{-6}	10	11
Flagellar Assembly	Depleted	6.72×10^{-9}	16	19
Bacterial Secretion System	Depleted	3.21×10^{-4}	13	17
RNA Degradation	Depleted	2.65×10^{-4}	4	8

was *fliT*, which is known to produce a hyperflagellated phenotype in deletion mutants (Yokoseki et al., 1995). It seems likely that other genes in this subsystem with expanding mutant populations produce similar paradoxical effects. Flagella have long been known to be important for *S.* Typhimurium infection of macrophage (Weinstein et al., 1984; Bäumler et al., 1994; Schmitt et al., 2001), and our results agree.

Bacterial secretion systems were also enriched in contracting mutant populations, see tables 3.3 and 3.4. Most prominent among these were SPI-1 and SPI-2 T3SSs, known to be involved in invasion of and survival in macrophage, respectively. SPI-2 genes had relatively low mutant prevalences in our initial library, likely due to the exclusion of transposase by the nucleoid-forming protein H-NS (see chapter 2); despite this SPI-2 genes are still enriched in contracting mutant populations, and the effect of SPI-2 genes on macrophage infection is likely underestimated by these results. Additionally this KEGG system does not include many of the effector proteins secreted by these T3SSs, so again their effect is likely to be underestimated. Finally, the RNA degradation system was also enriched in contracting mutant populations. The four genes in this system found to be significantly depleted in mutants after the infection assay were *pcnB*, involved in polyadenylation of transcripts; *hfq*, involved in the activity of bacterial sRNAs; *dnaK*, a molecular folding chaperone implicated in heat-shock responses; and *rnr*, encoding RNase R, a component of the bacterial RNA degradosome. This underlines the importance of RNA-based regulation to the infective process in *S.* Typhimurium, an emerging theme in infection biology (Hebrard et al., 2012).

Overall, the picture emerging from this high-level analysis of the *S.* Typhimurium macrophage infection assay recapitulates much of what is already know from the literature

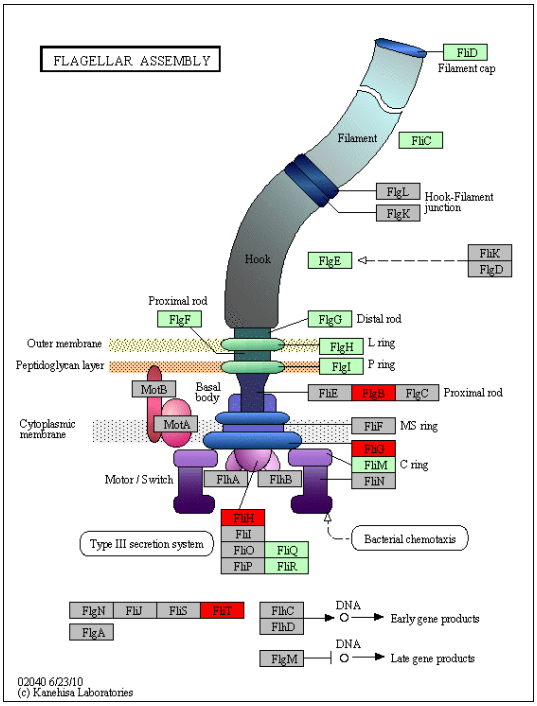


Figure 3.7: Mutant depletion in the *S.* Typhimurium flagellar subsystem. Genes in grey are relatively depleted in mutants over infection, while genes in red have mutant populations that have expanded. Figure adapted from the KEGG database (Kanehisa et al., 2012).

(see figure 3.1). It is an active process, involving flagella, manipulation of host cells through the SPI-1 and -2 T3SSs, and rapid RNA-based regulatory changes in response to the changing conditions during infection, and induces large changes in the population structure of our mutant library. In contrast, the structure of the *S.* Typhi library after infection is almost entirely unchanged (see figure 3.5). Perhaps most interestingly, the genes of the SPI-1 and -2 T3SSs displayed no significant differences in mutant prevalence, and were all significantly different in behavior from the same genes in *S.* Typhimurium (see table 3.4). This confirms a previous controversial study (Forest et al., 2010) which claimed that SPI-2 had no effect on *S.* Typhi survival in macrophage. In addition SPI-1 does not appear to have an appreciable effect on macrophage entry or survival, suggesting uptake through phagocytosis as a primary entry mechanism. As only 28 genes were significantly changed in mutant prevalence over the assay (table 3.5), I did not perform a pathway analysis and instead examined genes individually. A comparatively small

Table 3.4: Bacterial secretion system genes implicated in *S.* Typhimurium infection of macrophages. Genes in the KEGG bacterial secretion system category with statistically significant changes in mutant prevalence over macrophage infection. Columns: 1, SL1344 gene ID; 2, gene name; 3, SPI gene resides in; 4, logFC over *S.* Typhimurium infection of macrophage, negative values indicate a contraction of the mutant population, positive an expansion; 5, adjusted p-value for difference to *S.* Typhimurium control; 6, logFC between *S.* Typhi and *S.* Typhimurium experiments, negative values indicate faster contraction of the *S.* Typhimurium population and/or expansion of the *S.* Typhi population and vice versa; 7, p-value for difference from *S.* Typhi logFC.

SL ID	Name	SPI	logFC	P-value	Δ logFC	Δ P-value
SL1343	ssaJ	SPI-2	-4.65	1.29×10^{-7}	-4.83	1.79×10^{-8}
SL1355	ssaT	SPI-2	-4.46	1.41×10^{-4}	-5.04	7.55×10^{-6}
SL2868	spaQ	SPI-1	-4.10	2.37×10^{-6}	-3.95	5.55×10^{-6}
SL2650	ffh	N/A	-4.04	4.28×10^{-2}	-2.11	1.43×10^{-1}
SL2869	spaP	SPI-1	-3.30	3.75×10^{-15}	-3.09	1.80×10^{-11}
SL1352	ssaQ	SPI-2	-2.33	4.68×10^{-8}	-2.38	2.80×10^{-7}
SL1353	SL1353	SPI-2	-2.30	1.40×10^{-2}	-2.48	6.99×10^{-3}
SL1354	ssaS	SPI-2	-2.18	2.60×10^{-2}	-2.28	1.53×10^{-2}
SL2867	spaR	SPI-1	-1.93	2.50×10^{-7}	-1.84	6.16×10^{-6}
SL2853	prgI	SPI-1	-1.72	9.23×10^{-3}	-1.65	1.21×10^{-2}
SL2870	spaO	SPI-1	-1.70	2.62×10^{-5}	-1.91	9.31×10^{-6}
SL2876	invE	SPI-1	-1.67	1.36×10^{-3}	-1.61	2.80×10^{-3}
SL2873	SL2873	SPI-1	-1.25	9.00×10^{-3}	-1.12	2.01×10^{-2}
SL3928	tatB	N/A	1.45	5.99×10^{-2}	2.73	9.11×10^{-4}
SL1340	ssaG	SPI-2	1.51	7.97×10^{-2}	1.47	8.95×10^{-2}
SL1328	SL1328	SPI-2	1.82	9.68×10^{-2}	1.39	2.04×10^{-1}
SL3264	secG	N/A	2.67	6.16×10^{-3}	3.70	5.26×10^{-4}

number of genes appear to be actively selected for or against in the assay; however, this belies the broad effects these relatively few differences may have on the phenotypes exhibited by the population.

As in *S.* Typhimurium, a disproportionate number of genes with significant changes in mutant prevalence were involved in surface antigen and LPS biosynthesis: *rfaH*, *wecG*, *wecC*, *wecB*, *waaG*, *waaP*, *waaI*, and *waaJ*. However, in contrast to *S.* Typhimurium, many of the contracting mutant populations were in genes involved in biosynthesis of the enterobacterial common antigen (ECA). *S.* Typhimurium ECA mutants have previously

Table 3.5: Genes putatively involved in *S.* Typhi infection of macrophages. Genes with statistically significant changes in mutant prevalence over macrophage infection. See text for a discussion of gene function. Columns: 1, TY2 gene ID; 2, gene name; 3, logFC over *S.* Typhi infection of macrophage, negative values indicate a contraction of the mutant population, positive an expansion; 4, adjusted p-value for difference to *S.* Typhi control; 6, logFC between *S.* Typhi and *S.* Typhimurium experiments, negative values indicate faster contraction of the *S.* Typhimurium population and/or expansion of the *S.* Typhi population and vice versa; 7, p-value for difference from *S.* Typhimurium logFC.

Ty2 ID	Name	logFC	P-value	Δ logFC	Δ P-value
t0540	nuoF	-4.50	2.01×10^{-4}	4.79	8.23×10^{-4}
t1033	prc	-1.77	4.70×10^{-4}	-2.28	2.77×10^{-3}
t1038	yobG	-3.13	4.63×10^{-4}	-0.10	8.81×10^{-1}
t1662	hns	1.60	2.26×10^{-5}	-3.48	5.26×10^{-6}
t2312	t2312	1.64	9.41×10^{-6}	-3.37	2.87×10^{-9}
t2313	fimY	-1.49	2.30×10^{-4}	1.83	1.63×10^{-3}
t2317	fimD	-1.56	2.30×10^{-4}	2.13	4.02×10^{-4}
t2695	stpA	-1.56	2.30×10^{-4}	-3.08	2.81×10^{-7}
t2961	dsbC	-2.65	3.44×10^{-4}	2.90	3.85×10^{-3}
t2980	serA	-2.38	1.76×10^{-4}	4.04	2.17×10^{-6}
t3252	yhcH	2.05	2.60×10^{-11}	-2.96	2.05×10^{-11}
t3264	degQ	-1.48	1.25×10^{-4}	2.53	1.79×10^{-6}
t3320	rfaH	3.35	4.51×10^{-7}	-1.54	7.51×10^{-2}
t3368	wecG	-3.18	5.34×10^{-6}	2.40	5.56×10^{-3}
t3376	wecC	-1.59	4.63×10^{-4}	2.02	1.52×10^{-3}
t3377	wecB	-1.70	3.27×10^{-5}	0.50	3.67×10^{-1}
t3500	oxyR	1.92	4.70×10^{-4}	-1.73	2.98×10^{-2}
t3623	dsbA	-5.05	1.91×10^{-6}	6.66	9.26×10^{-8}
t3634	rbsK	-1.92	4.70×10^{-4}	2.36	2.30×10^{-3}
t3645	gidA	-2.89	4.50×10^{-10}	1.61	7.33×10^{-3}
t3677	mnmE	-2.15	7.61×10^{-7}	1.71	3.49×10^{-3}
t3796	waaG	-2.37	9.01×10^{-8}	6.28	1.22×10^{-27}
t3797	waaP	-3.36	2.37×10^{-7}	1.63	4.02×10^{-2}
t3801	waaI	3.41	1.99×10^{-36}	-1.60	1.34×10^{-5}
t3802	waaJ	1.37	1.36×10^{-4}	0.40	4.30×10^{-1}
t4179	actP	1.43	7.08×10^{-10}	-0.95	3.80×10^{-3}
t4411	miaA	-2.68	8.39×10^{-5}	1.25	1.29×10^{-1}
t4488	treC	-3.27	2.10×10^{-6}	3.91	1.97×10^{-6}

been shown to not cause acute disease in mice, though they are capable of persistently colonizing the spleen and liver (Gilbreath et al., 2012), reminiscent of *S.* Typhi infections of silent carriers, though the relevance of this to *S.* Typhi infection of macrophages is unclear. The most interesting of the LPS biosynthesis genes affected, *rfaH*, is a anti-termination factor affecting primarily LPS biosynthesis loci (Artsimovitch et al., 2002; Santangelo et al., 2002) with a >8-fold increase in mutant popluation size over the assay. This anti-termination factor associates with RNA polymerase and prevents pausing at both Rho-dependent and Rho-independent transcriptional terminators, promoting the expression of promoter-distal loci. As a result, a mutation in this single gene is likely to have broad pleiotropic effects, a feature common to many of the genes implicated in *S.* Typhi infection.

Other examples of genes with potentially wide-ranging pleiotropic effects include the paralogous nucleoid-forming proteins *hns* and *stpA*. H-NS has been described previously in chapter 2, but briefly it acts to condense DNA by binding to AT-rich, bent regions, and primarily regulates virulence and stress-response loci (Navarre et al., 2006; Lucchini et al., 2006). The paralogous StpA has similar binding affinity, but regulates a reduced regulon compared to H-NS, and in fact *hns* masks the phenotypic effects of an *stpA* deletion (Lucchini et al., 2009). The expansion of the population containing *hns* mutations with the concomitant contraction of *stpA* mutant populations suggests a subtle interplay between the two at work under infective conditions, with potentially wide repercussions for cellular phenotype. Another example of this theme of *S.* Typhi relying on genes with pleiotropic effects is given by *gidA* and *mnmE*. The products of these genes act together to post-transcriptionally modify a number of tRNAs (Yim et al., 2006), affecting translational fidelity (Brégeon et al., 2001). Mutations in these genes can have global effects (Kinscherf et al., 2002), and have recently been shown to affect *S.* Typhimurium virulence (Shippy et al., 2013).

While *hns*, *stpA*, *gidA*, and *mnmE* modulate gene expression at the transcriptional and post-transcriptional level, two other genes with depleted mutant populations, *dsbA* and *dsbC*, likely induce effects post-translationally. The *dsb* genes are involved in disulfide bond (DSB) formation, which is required for the proper folding and function of a wide range of proteins, and is known to be required for virulence in a number of bacterial species including *Shigella flexneri* and uropathogenic *Escherichia coli* (Heras et al., 2009). DsbA catalyzes the formation of DSBs in newly translated proteins translocated periplasm;

however, this process is non-specific and introduces spurious bonds. DsbC provides a proof-reading mechanism through isomerization of non-native bonds introduced by DsbA. This process is critical to the expression of a wide range of virulence factors in many species, including toxins and fimbriae (Yu et al., 1999). DsbA expression is known to affect *S. flexneri* survival in macrophage (Yu et al., 2001), and it appears to affect virulence in *S.* Typhimurium, though this is thought to be mediated through its effects on the SPI-2 T3SS (Miki et al., 2004). The exact mechanism through which the DSB system affects *S.* Typhi survival in macrophage is unclear, though it appears likely that DSB formation is important for extracellular or cell-surface structures *S.* Typhi uses to interact with the host cell.

In conclusion, the picture that emerges from this analysis is that unlike *S.* Typhimurium, *S.* Typhi is robust to assault from a human macrophage host cell. Infection produces only small changes in the population structure of the *S.* Typhi mutant library, and those populations which are affected have mutations in genes causing broad pleiotropic effects which can not help but have a strong effect on phenotype. This suggests that *S.* Typhi is already tuned to maintain homeostasis within human macrophages, indicative of its extreme adaptation to its host. While I have only performed a systematic analysis of the orthologous genes present in both *S.* Typhimurium and *S.* Typhi here, I have also examined the effect of macrophage infection on the genes involved in synthesis of the Vi antigen, which may be responsible for some of the differences exhibited between the two serovars. This capsular antigen confers a protective effect on *S.* Typhi in macrophage (Hirose et al., 1997), and as expected mutations in these genes were not well tolerated. It appears that *S.* Typhi, with the help of its capsule, adopts a stealth phenotype whereby it can enter and replicate within macrophage unmolested. *S.* Typhimurium, on the other hand, uses its flagella and SPI-encoded T3SSs to actively invade the macrophage, and the toll of this combat can be seen in the effects on the mutant population. I am currently working with Prof. John Wain (University of East Anglia) to procure microscopy of *S.* Typhimurium and *S.* Typhi infection of macrophage to confirm and build on these results.

While I have developed the methods presented in this chapter specifically to deal with this study, they are broadly generalizable to any transposon-insertion sequencing study. I am assisting in applying them to a number of TraDIS studies in a wide range of organisms, including carbon source utilization in *S.* Typhimurium and *S.* Enteriditis;

twitching motility in *Pseudomonas aeruginosa*; whole animal infection in *Citrobacter*, *Salmonella*, and *Escherichia* strains; and drug resistance in *Klebsiella pneumoniae*. As I have shown here, with the proper analytical tools TraDIS can be a powerful technique for the rapid generation of functional hypotheses about gene function in complex processes.

Chapter 4

Detecting Rho-independent terminators in genomic sequence with covariance models

Portions of this chapter are based on the previously published article "RNIE: genome-wide prediction of bacterial intrinsic terminators" (Gardner et al., 2011). This work is the result of collaboration with Paul P. Gardner (Wellcome Trust Sanger Institute/University of Canterbury).

4.1 Introduction

Bacteria are thought to utilize two major systems for transcriptional termination: Rho-dependent termination, and Rho-independent or intrinsic termination (Peters et al., 2011). Rho-dependent termination relies on a protein cofactor, Rho, a homohexameric ring protein that threads its way along the newly synthesized RNA molecule before causing RNA polymerase (RNAP) to dissociate at poorly defined pause sites. Intrinsic termination on the other hand, depends primarily on the biophysical characteristics of the sequence being transcribed. The detection of these intrinsic terminators in genomic sequence is the subject of this chapter. This chapter will serve largely as background and motivation for the next, in which I develop computational methods for identifying and characterizing transcriptional termination motifs across the bacterial phylogeny.

4.1.1 Rho-independent termination

Intrinsic termination is mediated by short structured RNA motifs known as Rho-independent terminators (RITs). These are generally characterized by a G+C-rich hairpin followed by a tract of T (as DNA) / U (as RNA) residues. As RNAP transcribes the poly-U tract it pauses, possibly with assistance from the partially formed hairpin structure, allowing full nucleation of the hairpin which melts weak rU-dA bonds within the elongation complex and leads to dissociation of RNAP (Peters et al., 2011), see figure 4.1. This process is somewhat stochastic, and the probability of successful transcription termination depends on various features of the RIT including stem composition and length, loop composition, length of the poly-U tract, and the sequence context of the element (Larson et al., 2008; Cambray et al., 2013; Chen et al., 2013). As is well known from the study of transcriptional attenuators and riboswitches (Henkin et al., 2002; Barrick et al., 2007; Naville et al., 2010), alternative structures formed upstream of the RIT can also affect termination efficiency, and force exerted on the upstream sequence can increase termination efficiency even in the absence of obvious alternative structures (Larson et al., 2008).

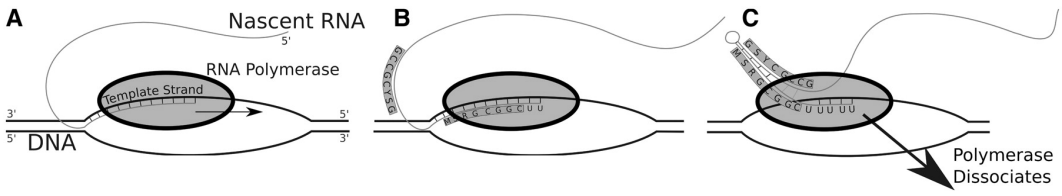


Figure 4.1: Rho-independent termination. A) The RNA polymerase traverses the DNA template strand from 3′ to 5′ , synthesizing the nascent RNA molecule. B) As the polymerase nears a termination site, a G+C-rich terminator stem sequence (boxed) is transcribed. C) Formation of a hairpin structure causes the polymerase to pause, and together with a string of unstable rU-dA bonds causes the polymerase to release from the template. Reproduced from Gardner et al. (2011).

The degree to which bacteria rely on intrinsic termination varies widely. A bioinformatic analysis examining the computationally predicted minimum free energy (MFE) of gene terminuses showed that while some species display an enrichment of strong RNA secondary structure potential at the 3′ ends of genes, others do not (Washio et al., 1998). Mutagenesis studies support this conculsion: while Rho is essential in some genomes

with fewer apparent intrinsic terminators (for instance, *Salmonella enterica*, see table 2.1), it is dispensable in others that are more heavily dependent on intrinsic termination, such as *Bacillus subtilis* (Quirk et al., 1993). This suggests competition between the two termination systems, leading to clade-specific skews in RIT utilization (Carafa et al., 1990; Kröger et al., 1998; Hoon et al., 2005). The accurate prediction of these elements is critical to understanding the regulation of transcription, particularly in light of the $\geq$3000 completed bacterial genomes currently deposited in EMBL-bank. In addition to their obvious role in helping to define operon structures in genomic sequence (Salgado et al., 2013), they can also be important indicators of cis-RNA regulation (Henkin et al., 2002; Barrick et al., 2007; Naville et al., 2010). Finally, the importance of RITs in designing synthetic genetic circuits has recently been recognized, and this has driven studies attempting to broaden our understanding of the factors affecting intrinsic termination efficiency (Cambray et al., 2013; Chen et al., 2013).

4.1.2 Previous approaches to identifying intrinsic terminators

Two main approaches to detecting RITs have been taken over the years: RNA motif descriptors, both expertly constructed (Lesnik et al., 2001) and automatically generated (Naville et al., 2011); and thermodynamic models of RNA folding to detect hairpins paired with a heuristic scoring scheme for the poly-T tail region (Ermolaeva et al., 2000; Wan et al., 2005; Wan et al., 2006; Kingsford et al., 2007). Arguably the most popular of these methods has been TransTermHP (Kingsford et al., 2007), an example of the second approach.

The TransTermHP algorithm takes a windowed approach to detecting RITs (figure 4.2). In order to avoid the computational cost of predicting local secondary structure across the entire genome, TransTermHP first scans overlapping windows of 6 bases for those containing at least 3 T residues. Upon finding such a window, TransTermHP performs a dynamic programming procedure to predict potential hairpin structures, using a simplified version of the Zuker algorithm (Zuker et al., 1981) for *in silico* RNA folding parameterized using a set of experimentally validated *Escherichia coli* RITs (Ermolaeva et al., 2000). This is then combined with a heuristic score for the quality of the poly-T tail (Carafa et al., 1990) which rewards T residues occurring closer to the closing base-pairs of the predicted hairpin structure. Candidate RITs are then filtered on stem length, loop

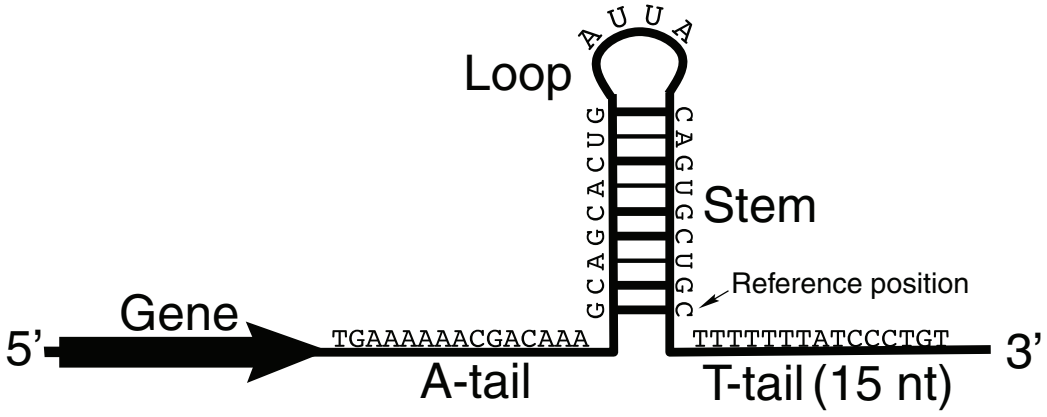


Figure 4.2: TransTermHP motif. Schematic of the terminator motif that TransTermHP searches for. The terminators consist of a short stem-loop hairpin followed by a thymine-rich region on their 3′ side. TransTermHP is generally restricted to find terminators where each side of the stem is ≥4 nt, the length of the loop is ≥3 nt and ≤13 nt, and the total length of the stem-loop is ≤59 nt. Reproduced under a Creative Commons Attribution License (CCAL) from Kingsford et al. (2007).

length, and total length (see the caption of figure 4.2 for details). Finally, the combined score of surviving candidates is compared to the scores of predicted terminators in random sequence with similar GC content to that of the target genome to provide a measure of prediction quality. Search is apparently also limited to regions surrounding stop codons (Kingsford et al., 2007; see also the discussion of the beta benchmark below), though the exact boundaries on the search space are not explicitly given in the TransTermHP documentation or publication.

This methodology presents a number of problems. First, while the thermodynamic method used to predict hairpin structures likely places some implicit restrictions on the sequence composition of the hairpin structure, it does not explicitly model conservation of residue composition across terminators. Conservation of residue composition could arise due to convergent evolution of terminator structures under selection for properties that promote strong termination in the host species, or as the results of genuine homology between RITs due to their descent from a common ancestor deposited by transposable elements, as has previously been hypothesized (Naville et al., 2010). In addition, windowed searching for and heuristic scoring of the poly-T tail is unlikely to accurately capture the true constraints on this feature. We show here that explicitly modelling residue

conservation improves detection of RITs. Secondly, the comparison to random sequence with similar GC content is unlikely to be an adequate control: it has been shown that considering dinucleotide content of sequences is critical to determining the significance of their secondary structure (Workman et al., 1999). Though the method of generating random sequence is not explicitly stated in Kingsford et al. (2007), it seems unlikely that it was the product of dinucleotide shuffling or a first-order Markov chain, as would be required to preserve dinucleotide frequencies. In fact TransTermHP does not appear to consider base-stacking effects in its predictions whatsoever. Finally, restricting search to the regions around annotated gene terminuses, or rewarding candidate RITs for being in these regions, is both somewhat artificial and requires accurate gene annotation, which remains a challenge.

4.1.3 Covariance models

Our method, RNIE, overcomes many of the problems in previous RIT detection methods through the use of *covariance models* (CMs), a special case of stochastic context-free grammars (Eddy et al., 1994; Sakakibara et al., 1994). CMs are sophisticated statistical models which incorporate information about both sequence and secondary structure conservation. They are perhaps most easily understood through analogy to the simpler profile hidden Markov models (HMMs) (Eddy, 1998). A typical method for HMM construction takes as its input an alignment of sequences. For each column of the alignment, a *node* is constructed, consisting of three *states*: match, insert, and delete. The match state models the residue distribution at that alignment position, while the insert and delete states model the probability and length distributions of insertions and deletions beginning at this column, respectively. The mathematics of HMMs have been well explored, and efficient dynamic programming algorithms exist for training (the Baum-Welch algorithm), assigning a probability to a sequence being produced by the model (the Forward algorithm), and finding the most probable parsing of a sequence (the Viterbi algorithm).

CMs are similar to profile HMMs, with the extension that they can additionally model dependence between alignment positions (see figure 4.3); rather than nodes being constructed from alignment columns, they are built from structural elements, i.e. pairing bases, annotated in the alignment (figure 4.3B). This increases the complexity of node

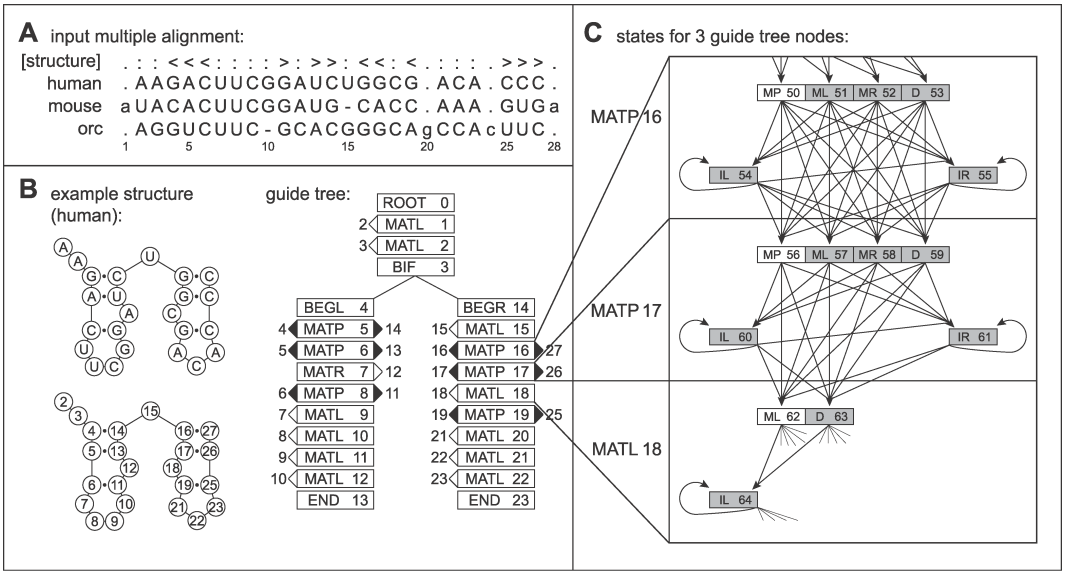


Figure 4.3: Covariance model architecture. A) A toy multiple alignment of three RNA sequences, with 28 total columns, 24 of which will be modeled as consensus positions. The [structure] line annotates the consensus secondary structure: angle brackets mark base pairs, colons mark consensus single-stranded positions, and periods mark insert columns that will not be considered part of the consensus model because more than half the sequences in these columns contain gaps. B) The structure of one sequence from A, the same structure with positions numbered according to alignment columns, and the guide tree of nodes corresponding to that structure, with alignment column indices assigned to nodes (for example, node 5, a MATP match-pair node, will model the consensus base pair between columns 4 and 14). C) The state topology of three selected nodes of the CM, for two MATP nodes and one consensus leftwise single residue bulge node (MATL, match-left). The consensus pair and singlet states (two MPs and one ML) are white, and the insertion/deletion states are gray. State transitions are indicated by arrows. Reproduced under a Creative Commons Attribution License (CCAL) from Nawrocki et al. (2007).

architecture (figure 4.3C), as each node must now contain states to match both bases in a pair, either one of a pair individually if its partner has been deleted, insertions on either side of the pair, and base pair deletions. Analogs to the Baum-Welch, Forward, and Viterbi algorithms exist for CMs: expectation-maximization using the inside-outside algorithm, the inside algorithm, and the Cocke-Younger-Kasami (CYK) algorithm, respectively. Unfortunately, modeling the dependence between positions, that is moving from a regular grammar such as an HMM to a context-free grammar such as a CM, comes at a considerable computational cost due to the restrictions imposed by the Chomsky

hierarchy (Chomsky, 1959), roughly equivalent to adding an additional dimension to the dynamic programming matrix. In this study we have used the Infernal package (Nawrocki et al., 2009), which implements CMs and associated algorithms for RNA sequence analysis, and includes a number of heuristics for increasing the speed of CM-based searches including adaptive banding of the dynamic programming matrix (Nawrocki et al., 2007) and HMM pre-filters based on HMMER (Eddy, 2011). Importantly, Infernal also incorporates a null model for scoring sequence hits; for sequence that matches the CM, the probability of this match is compared to the probability of a match to the null model. This comparison is expressed as a log_2 odds ratio, or bitscore, and from this further statistics, such as an expect value (E-value), can be calculated. Covariance models are widely used in RNA homology search, most notably by the Rfam database (Burge et al., 2013) and the tRNAscan-SE tool (Lowe et al., 1997) for predicting tRNAs in genomic sequence.

4.2 Methods

Paul P. Gardner implemented and benchmarked the RNIE tool. Eric P. Nawrocki (Howard Hughes Medical Institute Janelia Farm Research Campus) assisted in optimizing Infernal parameters for RIT search. Zasha Weinberg (Howard Hughes Medical Institute/Yale University) ran the Rnall and Rnall-Brkr algorithms for benchmarking. I designed and implemented the analysis which lead to the discovery of the putative mycobacterial termination motif.

4.2.1 Construction of a covariance model for Rho-independent terminators

One hundred seventy-one and 891 experimentally validated RIT sequences from *Escherichia coli* and *Bacillus subtilis*, respectively, were collected from the *E. coli* Database Collection (ECDC; Wahl et al., 1995) and the supplementary information of Hoon et al. (2005) and manually curated based on evidence quality, leaving a set of 981 RIT sequences. These sequences were subjected to iterative rounds of alignment, structure prediction, homology search and refinement. Alignments and secondary structures were predicted using WAR (Torarinsson et al., 2008a), CMfinder (Yao et al., 2006), and MLocarna (Will

et al., 2007), iteratively refined using Infernal (Nawrocki et al., 2009), then manually refined using the RALEE emacs environment (Griffiths-Jones, 2005). Sequence searches were performed using the resulting CM against EMBL with the Rfam pipeline (Gardner et al., 2009), and additional sequences were incorporated in to the alignment based on the following criteria: i) the maximum similarity to an existing seed sequence had to be 95% and the minimum 60%, ii) the minimum fraction of canonical base pairs had to be 75%, iii) the sequence annotation should not contain terms like contaminant, pseudogene, repeat or transposon and iv) they must score above a bitscore threshold of 20. These additional sequences were then manually checked for their position near a gene terminus. This resulted in 1117 aligned sequences, which were further split in to two groups based on how well they matched the resulting CM. Those scoring with a bitscore over 14 were placed in alignment A, those scoring less were placed in alignment B. These alignments were then again automatically refined using Infernal before a final round of manual refinement.

4.2.2 RNIE run modes

As described in the introduction, algorithms for performing inference with CMs can be very slow, and as a result Infernal implements a number of filters to reduce the number of sequences which proceed to a full CM-based homology search. In response to this, two modes for RNIE were developed: genome mode meant for large-scale searches, which enables HMM filters and adaptive banding and uses the CYK algorithm with a higher threshold for reported RIT predictions; and gene mode meant for annotation of relatively short sequence regions, which disables Infernal's filtering mechanisms and uses the slower but more powerful inside algorithm with a lower threshold for reporting RIT predictions. Genome mode scans sequence at ∼43 kb/s with a low false positive rate of ∼1.7 FP/Mb. The sensitivity, positive predictive value and Matthews' correlation coefficient for this mode (determined in the alpha benchmark below) are 0.70, 0.79 and 0.74. Gene mode scans at ∼1kb/s, and the false positive rate, positive predictive value and Matthews' correlation coefficient are ∼9.6 FP/Mb, 0.45 and 0.61, respectively. The Infernal parameters used for genome and gene mode, respectively, are

```
cmsearch -T 16 -g --fil-no-qdb --fil-T-hmm 2
--cyk --beta 0.05 CM query_sequence.fasta
```

```
cmsearch -T 14 -g --fil-no-qdb --fil-no-hmm
--no-qdb --inside CM query_sequence.fasta
```

4.2.3 Definitions

For the purposes of benchmarking, the following measures were used

$$Sensitivity = \frac{TP}{TP + FN}$$

$$PPV = \frac{TP}{TP + FP}$$

$$FPR = \frac{FP}{\text{Total length in kb}}$$

$$MCC = \frac{TP \times TN - FP \times FN}{\sqrt{(TP + FP)(TP + FN)(TN + FP)(TN + FN)}}$$

where any prediction that covered a known RIT by at least one nucleotide was considered a true positive (TP), any prediction that did not overlap a known RIT was considered a false positive (FP), a missed RIT was considered a false negative (FN), and the number of unclassified, non-RIT sequence were considered true negatives (TN).

Table 4.1: Control genomes. Columns: 1) species name, 2) EMBL-bank accession, 3) phylum, 4) genome size in megabases, 5) number of CDSs annotated in genome, 6) genome G+C content, 7) number of RNIE predictions in genome mode on native sequence, 8) number of RNIE predictions in genome mode on dinucleotide shuffled sequence, 9) number of RNIE predictions in gene mode on native sequence, 10) number of RNIE predictions in gene mode on dinucleotide shuffled sequence.

Species	EMBL accession	Phylum	Genome size (MB)	CDSs	G+C content	Number of predictions			
						Genome		Gene	
						native	shuffled	native	shuffled
Mycobacterium tuberculosis	AE000516	Actinobacteria	4.40	4189	0.66	19	0	111	3
Streptomyces griseus	AP009493	Actinobacteria	8.55	7138	0.72	72	0	353	2
Bacteroides thetaiotaomicron	AE015928	Bacteroidetes	6.26	4778	0.43	783	2	1470	44
Chlamydophila pneumoniae	AE001363	Chlamydiae	1.23	1052	0.41	61	3	135	19
Prochlorococcus marinus	AE017126	Cyanobacteria	1.75	1882	0.36	81	5	131	22
Deinococcus radiodurans	AE000513	Deinococcus-Thermus	2.65	2579	0.67	283	0	506	2
Bacillus subtilis	AL009126	Firmicutes	4.22	4245	0.44	1851	4	2540	54
Clostridium difficile	AM180355	Firmicutes	4.29	3777	0.29	431	8	1152	58
Fusobacterium nucleatum	AE009951	Fusobacteria	2.17	2067	0.27	155	1	457	34
Thermodesulfovibrio yellowstonii	CP001147	Nitrospirae	2.00	2033	0.34	78	6	176	41
Escherichia coli	U00096	Proteobacteria	4.64	4321	0.51	601	6	1058	35
Helicobacter pylori	AE000511	Proteobacteria	1.67	1566	0.39	28	12	128	61
Salmonella enterica	AE014613	Proteobacteria	4.79	4323	0.52	537	4	980	32
Leptospira interrogans	AE016823	Spirochaetes	4.28	3394	0.35	164	18	375	132
Ureaplasma parvum	AF222894	Tenericutes	0.75	611	0.26	54	0	163	5
Fervidobacterium nodosum	CP000771	Thermotogae	1.95	1750	0.35	409	3	588	28
Methylacidiphilum infernorum	CP000975	Verrucomicrobia	2.29	2472	0.45	50	7	157	52

4.3 Results

Benchmarking a tool for RIT detection is challenging. As described in the methods section, only a relatively small number of RITs had been verified at the time of this study. While this situation is beginning to improve with the development of high-throughput techniques for RIT characterization (Cambray et al., 2013; Chen et al., 2013), verified RITs are still largely drawn from the model bacteria *E. coli* and *B. subtilis*. As a result, two benchmarks were performed: the first, or alpha, benchmark examines method performance on known RITs, with the caveat that these RITs formed part of the training set for RNIE and many of the other methods tested. The second, or beta, benchmark is a qualitative assessment on whole genomes with unknown RIT contents, evaluating the quality of predictions by their genomic position and estimating the FPR by the relative number of predictions on shuffled sequence.

4.3.1 Alpha benchmark

For the first benchmark 485 known RITs, curated on the basis of experimental evidence for activity, were used, drawn from the ECDC (Wahl et al., 1995) and the supplementary information of Hoon et al. (2005). Each RIT was embedded in 1000 bases of randomly selected dinucleotide shuffled sequence drawn from the genomes in table 4.1. For each known RIT a first-order Markov chain was trained on the nucleotide distribution of that sequence and 100 decoy sequences were generated and similarly embedded in 1000 bases of dinucleotide shuffled sequence. A first-order Markov chain was used rather than dinucleotide shuffling of the native RITs, as these short sequences may have a limited number of permutations with identical dinucleotide content. As TransTermHP will only run on annotated sequence, artificial gene annotations were added to each sequence, with either decoys or genuine RITs positioned at the 3′ end of one of the annotations.

Four methods besides RNIE were tested (figure 4.4): TransTermHP (with 2, 4, 9, or 10 gene features; Kingsford et al., 2007), RNAmotif (using either the structural score alone (struct), or the structural score augmented with a score for hybridization between the poly-U tail and the DNA sequence (dG); Lesnik et al., 2001), Rnall (using either the score for hairpin formation (dG), or the score for hairpin formation augmented with a score for poly-U/DNA hybridization (hbG); Wan et al., 2005; Wan et al., 2006), and a

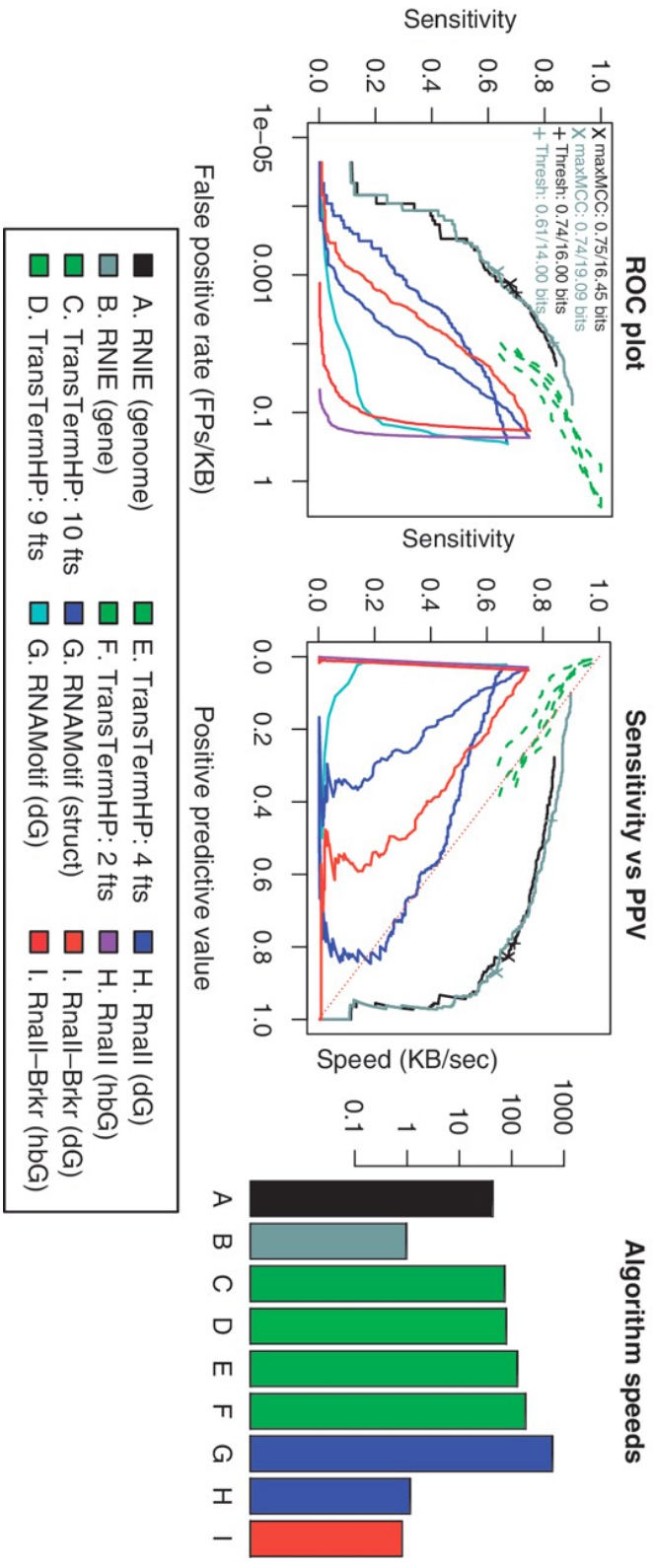
version of Rnall modified by the Breaker lab at Yale Univeristy (Rnall-Brkr; using either the score for hairpin formation (dG), or the score for hairpin formation augmented with a score for poly-U/DNA hybridization (hbG); Barrick et al., 2007; Weinberg et al., 2007).

The results of this benchmark show that RNIE's performance is superior to any previous method for detecting RITs at any level of sensitivity or specificity. Interestingly, all methods which rely on poly-U/DNA hybridization scores performed extremely poorly, suggesting that the understanding of the role of RNA-DNA hybridization in intrinsic termination modelled by these methods is incorrect, or at best incomplete. Of the other methods, the only ones besides RNIE which cross the line $y = 1 - x$, the performance of a hypothetical 'random' predictor, on the sensitivity versus PPV plot were TransTermHP and RNAmotif. The scanning speed of RNIE in genome mode, ∼43 kb/s, is comparable to that of TransTermHP at ∼74-186 kb/s, depending on the number of gene annotations. Based on these results, thresholds were chosen for reporting RNIE RIT predictions in genome and gene modes at levels slightly below the maximum MCC, that is allowing for a slightly higher FPR in return for increased sensitivity with the assumption that false positives can often be determined by their genomic context.

4.3.2 Beta benchmark

For the second benchmark 17 genomes representative of the diversity of the bacterial phylogeny (table 4.1) were scanned with both RNIE and TransTermHP, and the results compared. Additionally, dinucleotide shuffles of these genomes were scanned to provide an estimate of the FPR of each method. Genuine RITs are expected to occur preferentially in the 3′ region of annotated genes. As can be seen in figure 4.5, predictions for both RNIE and TransTermHP are enriched in predictions 3′ to gene annotations (solid lines). RNIE makes relatively few predictions in shuffled sequence (dashed lines), particularly in the more stringent genome mode, and these appear to be randomly distributed with respect to gene terminuses. Worryingly, TransTermHP predictions on dinucleotide shuffled

Figure 4.4 ***(following page)*****: Alpha benchmark.** The accuracy of RNIE compared to existing methods of terminator prediction. The left figure shows a ROC plot for four independent methods. The middle figure compares the sensitivity and PPV for the four methods. The figure on the right shows the speeds for each algorithm in kilobases per second. Reproduced from Gardner et al. (2011).


ROC plot
Sensitivity
False positive rate (FPs/KB)
maxMCC: 0.75/16.45 bits
maxMCC: 0.74/19.09 bits
Thresh: 0.74/16.00 bits
Thresh: 0.61/14.00 bits
Sensitivity vs PPV
Sensitivity
Positive predictive value
Algorithm speeds
Speed (KB/sec)
A. RNIE (genome)
B. RNIE (gene)
C. TransTermHP: 10 fts
D. TransTermHP: 9 fts
E. TransTermHP: 4 fts
F. TransTermHP: 2 fts
G. RNAMotif (struct)
G. RNAMotif (dG)
H. RnaII (dG)
H. RnaII (hbG)
I. RnaII–Brkr (dG)
I. RnaII–Brkr (hbG)

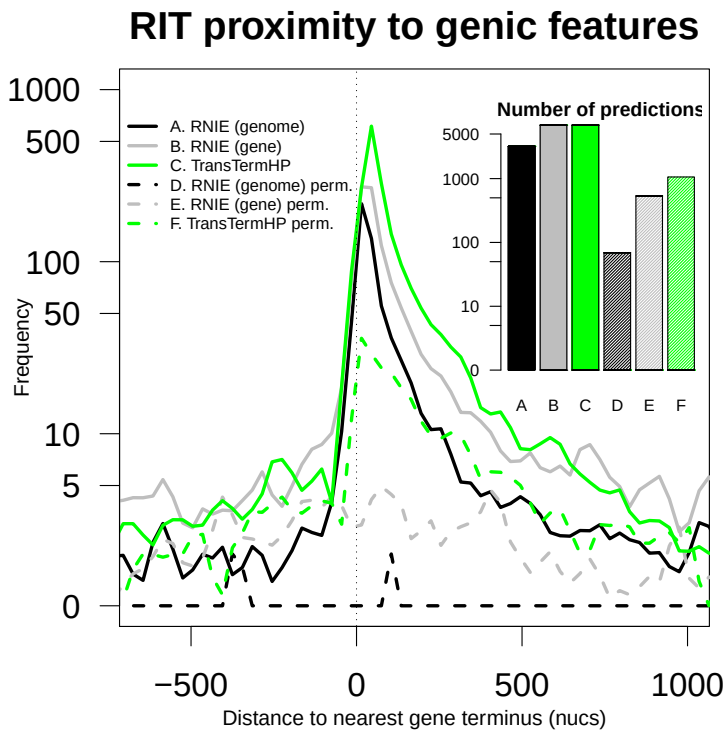
sequence are also enriched at annotated gene terminus, suggesting it is giving a bonus to predictions falling in the correct genomic context. This is particularly problematic, as it suggests a higher FPR in regions where RIT predictions will look most reasonable on a passing inspection.

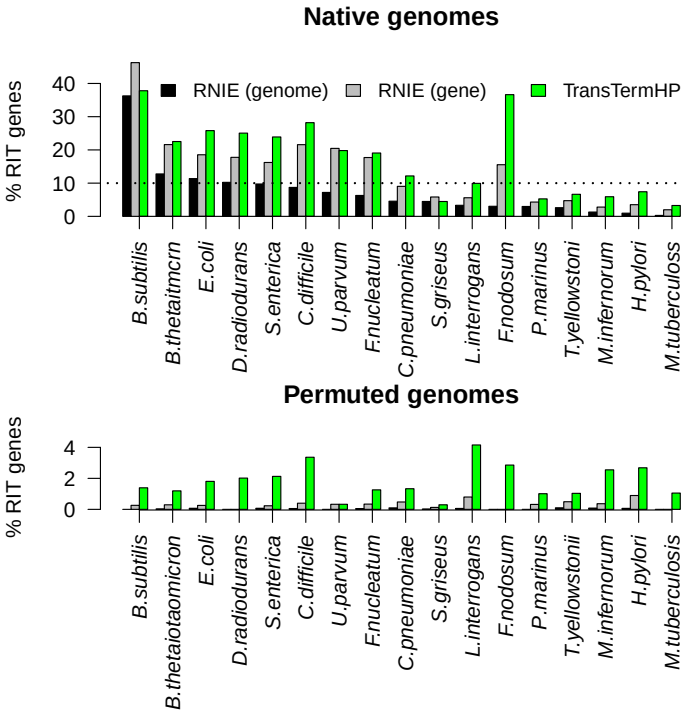
The bar plots in figure 4.5 report the percentage of genes reported to be terminated by a RIT in each genome by TransTermHP and RNIE. In general, the number of predictions made by RNIE is comparable to TransTermHP, particularly when the higher number of predictions by TransTermHP on shuffled sequence is taken in to account. Interestingly, the only genome where RNIE predicts more RITs than TransTermHP is *B. subtilis*, where most of the training data for the RNIE CMs originated. Additionally, there are a number of genomes where few RITs are predicted by either method. Both of these points will be addressed in more detail in the next chapter.

4.3.3 A novel termination motif in *Mycobacterium tuberculosis*

In the course of benchmarking RNIE, we noticed that neither our method nor TransTermHP made many RIT predictions in the *Mycobacterium tuberculosis* genome. While some bacterial lineages are hypothesized not to use intrinsic termination, there is a body of prior work suggesting that *M. tuberculosis* does utilize secondary structure in termination (Washio et al., 1998; Unniraman et al., 2001; Unniraman et al., 2002; Mitra et al., 2008; Mitra et al., 2009). In particular the Nagaraja group has developed a method, GeSTer,

Figure 4.5 ***(following page)*****: Beta benchmark.** Ideal terminator predictors will generally produce predictions that are immediately 3′ to annotated genes on native sequence and no predictions on shuffled controls. For all the test genomes in Table 1 (excluding *E. coli* and *B. subtilis*), we computed the distance to the nearest 3′ genic element, including CDSs, ncRNAs and riboswitches. This was done for both native sequences and dinucleotide shuffled control sequences with corresponding gene annotation transferred to the controls. The figure on the left shows the distribution of distances for RNIE genome and gene modes and for the TransTermHP method. Inset is a barplot showing the total number of predictions for each method on native and shuffled genomes. The figures on the right show the percentage of genes that have a predicted RIT in the region −50 to +150 from an annotated 3′-end of a CDS or ncRNA across all the genome sequences described in Table 1. The upper panel illustrates the results for the native genomes, while the lower panel illustrates results for the permuted genomes. Reproduced from Gardner et al. (2011).


RIT proximity to genic features
A. RNIE (genome)
B. RNIE (gene)
C. TransTermHP
D. RNIE (genome) perm.
E. RNIE (gene) perm.
F. TransTermHP perm.
Number of predictions
5000
1000
100
10
0
A B C D E F
Frequency
1000
500
100
50
10
5
0
−500
0
500
1000
Distance to nearest gene terminus (nucs)


Native genomes
RNIE (genome)
RNIE (gene)
TransTermHP
% RIT genes
40
30
20
10
0
B.subtilis
B.thetaitmcrn
E.coli
D.radiodurans
S.enterica
C.difficile
U.parvum
F.nucleatum
C.pneumoniae
S.griseus
L.interrogans
F.nodosum
P.marinus
T.yellowstoni
M.infernorum
H.pylori
M.tuberculoss
Permuted genomes
% RIT genes
4
2
0
B.subtilis
B.thetaiotaomicron
E.coli
D.radiodurans
S.enterica
C.difficile
U.parvum
F.nucleatum
C.pneumoniae
S.griseus
L.interrogans
F.nodosum
P.marinus
T.yellowstonii
M.infernorum
H.pylori
M.tuberculosis

which attempts to classify predicted secondary structures from the terminuses of coding regions in to one of five categories of structural motifs. More than 90% of terminal motifs in *M. tuberculosis* fall in to their "I-shaped" category, or short stem-loop with no poly-U tail. With this in mind, I developed the following procedure to search for a potential structured termination motif in *M. tuberculosis*.

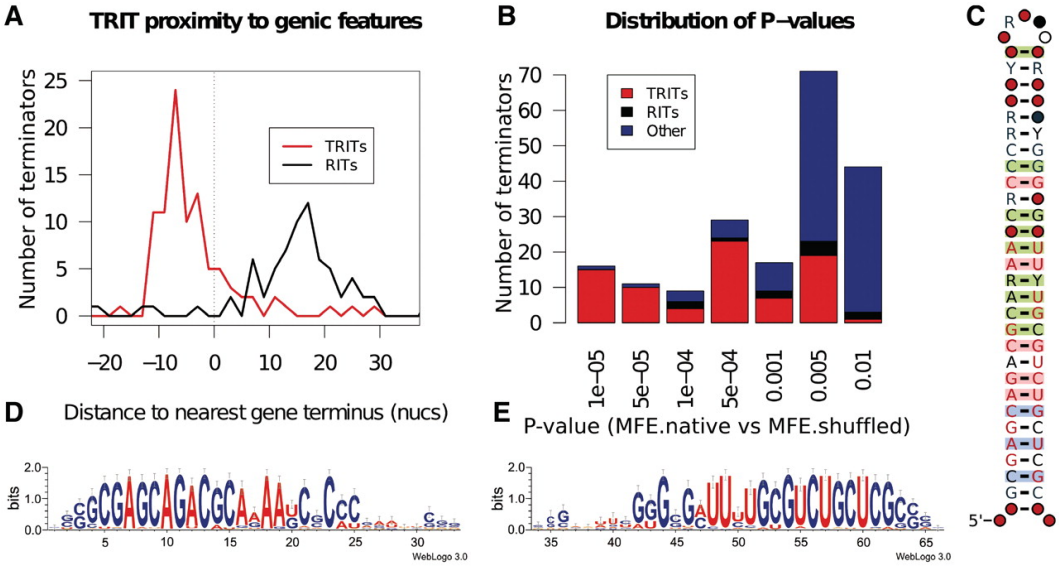


Figure 4.6: Putative mycobacterial transcription termination motif. A) The frequency of TRITs and RITs near the terminal regions of *M. tuberculosis* (EMBL accession: AE000516) genic features. B) The distribution of structural stability derived p-values for the most significant *M. tuberculosis* terminal regions coloured by TRIT (red), RIT (black) or unclassified (blue). C) The secondary structure and sequence conservation of the TRIT motif as displayed by R2R (Weinberg et al., 2011). (D&E) Sequence logos generated for the 5′ D) and 3′ E) halves of an alignment of the 147 copies of TRIT in the *M. tuberculosis* genome. Reproduced from Gardner et al. (2011).

I extracted 100-nucleotide 3′ sequences from the *Mycobacterium tuberculosis* CDC1551, starting 20 bases before annotated CDS ends. Predicted MFE folding scores for each sequence were calculated using RNAfold (Hofacker et al., 1994). I performed a pooled permutation test for lower than expected MFEs using 1000 dinucleotide shuffles from each 3′ sequence. I then ran the CMfinder (Yao et al., 2006) RNA motif-finder over sequences with a p-value less than 0.001. The subsequent alignment was manually refined using the RALEE RNA alignment editor (Griffiths-Jones, 2005). The refined alignment

was used to construct an Infernal CM (Nawrocki et al., 2009), as had been done for canonical RITs, which was then searched across all Mycobacteria genomes in the EMBL nucleotide sequence database.

This revealed a well-conserved structured sequence motif associated with gene terminal regions in Mycobacteria which we named the tuberculosis Rho-independent terminators, or TRITs, in light of the source of the discovery (see Figure 4.6). TRITs are found across the entire genus, ranging in approximate copy-number from 150 to 250 in *M. abscessus*, *M. avium*, *M. bovis*, *M. gilvum*, *M intracellulare*, *M. kansasii*, *M. leprae*, *M. marinum*, *M. smegmatis*, *M. tuberculosis*, *M. ulcerans* and *M. vanbaalenii*. The TRITs account for 72% (59/82) of terminal sequences with highly significant secondary structure ($p < 0.001$) in *M. tuberculosis*. TRIT predictions made by our model fall overwhelmingly at the terminus of annotated coding regions, tending to start 8 bases before the annotated gene end (Figure 4.6A), distinct from the distribution of RITs. In addition, TRITs appear to be associated with sharp drops in transcription in RNA-seq experiments (data presented in the next chapter). Additionally, since the publication of this study two sRNA screens in Mycobacteria have discovered TRITs apparently terminating sRNA transcription (Miotto et al., 2012; Li et al., 2013), providing additional evidence for their activity. The high sequence conservation (Figure 4.6D&E) across elements suggests that this element has either arisen relatively recently, or possibly requires a nucleotide-binding co-factor to perform its function. In the next chapter, I describe a study scaling up this approach to discover transcriptional termination motifs across the entire bacterial phylogeny.

TAM

Chapter 5

Kingdom-wide discovery of bacterial intrinsic termination motifs

5.1 Introduction

As discussed in the previous chapter, intrinsic termination of transcription is a fundamental cellular process in many, if not all, bacterial species. As reviewed in the previous chapter, the bulk of work on intrinsic termination has focused on canonical Rho-independent terminators (RITs), consisting of a G/C-rich hairpin structure followed by a poly-U tail. This is due to both their prevalence in model organisms such as *Escherichia coli* and *Bacillus subtilis*, as well as the distinctiveness of this motif making it an easy target for automated classification.

Despite this focus on canonical RITs, a number of intrinsic terminators which do not rely on a poly-U tail for termination activity are known. These include synthetic constructs derived from canonical RITs (Abe et al., 1996), as well as naturally occurring terminators in *Streptomyces* (Deng et al., 1987; Neal et al., 1991; Ingham et al., 1995) and Mycobacteria (Unniraman et al., 2001). Additionally, a number of ncRNA screens in Actinobacteria have described potential non-canonical RITs terminating ncRNA transcription (Swiercz et al., 2008; Miotto et al., 2012; Li et al., 2013). However, a more wide-spread effort at characterization of these elements has been hampered by two factors: their occurrence primarily in non-model organisms such as the Actinobacteria, and a lack of a systematic classification of these elements making it difficult to determine how

wide-spread such elements are. The only study surveying potential alternative intrinsic terminators in the bacterial kingdom relied primarily on categorizing elements based on the shape of their predicted secondary structure (Unniraman et al., 2002). However, this fails to consider the large number of very different sequences that can give rise to any particular secondary structure (Schuster et al., 1994). It is well understood from studies of synthetic perturbations of canonical RITs that the sequence of both the hairpin structure and flanking sequence can have large, and often unexpected, effects on termination efficiency (Reynolds et al., 1992; Abe et al., 1996; Cambray et al., 2013; Chen et al., 2013); there is no reason to think that non-canonical RITs would not exhibit a similar pattern of sequence specificity. As a result, there is a need for a robust classification of potential non-canonical RITs which considers both the sequence and structural features of these elements so that they can be systematically investigated.

In the previous chapter I showed that covariance models (CMs) are able to capture sequence as well as structural features of canonical as well as putative non-canonical RITs. In this chapter I describe a method for the discovery of potential structured termination motifs across the bacterial kingdom, present an initial analysis of the elements discovered, and provide evidence for their activity through the analysis of a large collection of publicly-available RNA-seq data.

5.2 Methods

James Hadfield (University of Canterbury) ran the MCL clustering under my supervision. Paul P. Gardner (University of Canterbury) developed and ran the analysis of expression data, and assisted in manual curation of cluster alignments. Stinus Lindgreen (University of Canterbury/University of Copenhagen) processed RNA-seq data and performed mapping. I performed all other work described here.

5.2.1 Genome-wise motif discovery

1853 EMBL format files containing the genomic sequence and annotations for 1639 bacteria were obtained from the EMBL European Nucleotide Archive completed bacterial genomes pages, see Appendix B for organisms and accession numbers.

Each EMBL file was screened independently for putative multi-copy termination

motifs. For each EMBL file, I extracted sequences from -20 to +80 around annotated ORF stop site. Each extracted sequence was screened for a lower than expected predicted MFE using RNAfold in order to screen out locally GC-rich but unstructured sequences. The sequence under consideration was shuffled 1000 times preserving dinucleotide frequencies, and a Gumbel distribution was fitted to the resulting empirical null MFE distribution using the R MASS package (Venables et al., 1994). Sequences with a native MFE below the 95th percentile of the null distribution were discarded. The resulting set of sequences was then given as input to CMfinder (Yao et al., 2006), which produces collections of locally-aligned structurally conserved motifs. I built a CM for each motif using Infernal 1.0.2 (Nawrocki et al., 2009). The resulting CMs were searched against the EMBL file the motif was discovered in, and were then screened on the following criteria for the collection of search hits with an E-value of less than 1: a copy number of between 100 and 3000, and a median distance of <10 to the nearest annotated ORF stop site. This resulted in a collection of 4359 putative termination motif CMs, each derived from a single EMBL file.

5.2.2 Clustering covariance models

In order to cluster CMs, I developed an extension of MCL-based clustering (Enright et al., 2002) to generative models of sequence variation. I call the measure of CM similarity I developed for this purpose the reciprocal similarity score (RSS), defined as:

$$\left[\frac{\sum_{i=1}^{n} -\ln\left(E_{x,y,i}\right) + \sum_{j=1}^{n} -\ln\left(E_{x,y,j}\right)}{2n}\right] + \ln(n)$$

where $E_{x,y,i}$ is the E-value of the ith sequence emitted by model x scored by model y and for the purposes of this study $n = 1000$. Briefly, for each pair of CMs 1000 sequences were emitted from each CM and reciprocally scored with the other CM. The average of the negative log-transformed E-values was calculated, then shifted to be strictly positive by adding ln(1000) to generate the RSS appropriate for use with MCL. MCL was run over the resulting RSS matrix, and the 100 largest clusters, ranging in size from 332 to 6 CMs, were taken forward for further analysis.

5.2.3 Building consensus covariance models

To build covariance models which captured the diversity of sequences represented by each cluster, I searched the ten CMs with the highest sum of RSS scores in each cluster against the set of genomes which contributed motifs to the cluster. Sequences on which at least four CMs agreed on with an E-value of < 1 were collected. The redundancy of the collected sequences was iteratively reduced in an alignment-free fashion using cd-hit (Li et al., 2006) with the parameters -G 0 -aL 0.1 -aS 0.3 until there were less than 2000 sequences remaining or there were no remaining sequences with $> 85\%$ nucleotide identity. Sequences were extended by 20 bases on each side to capture features which may not have been in the CMfinder-derived motifs, e.g. poorly conserved poly-U tracts. The resulting set of sequences was aligned using MAFFT Q-INS-i (Katoh et al., 2008) using McCaskill base-pairing probabilities (McCaskill, 1990), and secondary structures were predicted using CentroidAlifold (Hamada et al., 2009), again with McCaskill base-pairing probabilities. CMs were built from the resulting cluster alignments, and sequences which did not match the CM with a bitscore of at least 20 were iteratively discarded. The resulting alignments were then manually curated using RALEE (Griffiths-Jones, 2005), trimming non-conserved flanking sequence and extending the predicted secondary structure where possible. Conserved stop codons were specifically trimmed, so as not to bias subsequent searches.

5.2.4 Genome annotation

The resulting cluster CMs were searched over the initial 1853 EMBL files. Bitscore thresholds were set for hit significance for each cluster CM using shuffled sequence. Specifically, each cluster model was also used to search a dinucleotide shuffled database of these same 1853 EMBL file. For each model, a Gumbel distribution was fitted to the distribution of bitscores over the shuffled database, and this null Gumbel distribution was used to compute P-values for hit significance in the native sequences. P-values were corrected for multiple hypothesis testing using the method of Benjamini et al. (1995), and these were used to set bitscore thresholds at specific FDRs reported in the text, generally 1% or 5%.

5.2.5 Analysis of expression data

Data sets were downloaded from the SRA (Leinonen et al., 2011), preferring whenever possible to start our own analyses with the raw fastq input instead of relying on previous mapping results. This was done to make the data sets comparable. After retrieving the data sets, we extracted fastq reads for further analysis. Most data sets were downloaded in SRA format. Fastq files were extracted using the command fastqdump –split-3 from the SRA toolkit version 2.3.2-4. This creates two fastq files in the case of paired end data, and one fastq file in case of single end data. When BED files were used as the primary input, the BAM file was extracted directly using bedToBam from the bedtools package, version 2.17.0 (Quinlan et al., 2010). Data sets in SOLiD format was translated to fastq using solid2fastq from bfast version 0.7.0a (Homer et al., 2009). All extracted fastq files were cleaned using AdapterRemoval version 1.4 (Lindgreen, 2012) with the flags –trimns –trimqualities to remove residual adapters from the reads and to remove low quality segments and stretches of Ns in the 5′ and/or 3′ ends.

Most data sets were mapped using bowtie2 version 2.1 (Langmead et al., 2012), and the output was saved in BAM format using samtools version 0.1.18 (Li et al., 2009). In the single end case, the following command was used:

```
bowtie2 -x <INDEX> -U <READS> |samtools view -bS - \
| samtools sort - <OUTPUT>.sorted
```

In the paired end case, a similar command was used, but the number of input files was larger because 1) there are two files containing the paired reads, and 2) additional single end reads might have been produced by AdapterRemoval because some pairs were collapsed due to overlaps, or one mate pair was discarded due to e.g. low quality. For 454 data, using the above command produced few mappings to the reference genome. We therefore used bowtie2 but with relaxed parameters to accommodate the longer reads by adding the flags –local –very-sensitive-local. For SOLiD data, we used bfast version 0.7.0a for mapping with the following commands:

```
bfast match -f <INDEX> -A 1 -r <READS> > <OUTPUT>.bmf
bfast localalign -f <INDEX> -A 1 -m <OUTPUT>.bmf > <OUTPUT>.baf
bfast postprocess -f <INDEX> -i <OUTPUT>.baf -A 1 | samtools view -bS - \
| samtools sort - <OUTPUT>.sorted
```

For each BAM-file, we generated a PLOT file containing two tab separated columns (reverse strand, forward strand) and a line per position in the genome. Each line gives information on the number of mapped reads on each strand for that particular position in the reference genome. The PLOT files were generated using the following commands from samtools version 0.1.18:

```
samtools view -F 0x10 -b <INPUT> (for reads mapped to the forward strand)
samtools view -f 0x10 -b <INPUT> (for reads mapped to the reverse strand)
```

Then, the samtools depth command was used to get the actual depths and save them in a WIG format file, which was then transformed to PLOT file by filling out the 0-depth positions based on the length of the reference genome.

Terminator activity plots were produced by selecting all predicted putative attenuation motifs (TAMs) at an FDR of 5% with an upstream mean read count of at least 10. The median expression at each position between -80 and +80 with respect to the TAM was calculated and plotted. As a negative control, random positions meeting the criteria of a mean upsteam read count ≥ 10 were selected at random and their median recounts plotted. Specific data sets are cited in the text.

5.3 Results

5.3.1 Kingdom-wide motif discovery

The pipeline I developed for discovering putative termination motifs consisted of 3 major stages: genome-wise motif discovery with CMfinder (Yao et al., 2006), clustering of motifs using a novel similarity measure and the MCL algorithm (Enright et al., 2002), and manual curation of the resulting motif clusters.

In the first stage I extracted sequence from -20 to +80 with respect to annotated stop sites, which were then filtered on predicted structural potential to screen for sequences with stronger structures than predicted by their dinucleotide content alone (see Methods). For each genome, I used the resulting set of sequences as input for the CMfinder alogrithm (Yao et al., 2006). Briefly, CMfinder uses heuristic sequence search, thermodynamic and mutual information-based predictions of secondary structure, and CM-based searches within an expectation-maximization (EM) framework to iteratively

discover and refine potential structured RNA motifs, returning a multiple sequence alignment and corresponding CM. CMfinder has previously been successfully used as part of pipelines for the discovery of non-coding RNAs in bacteria (Weinberg et al., 2007; Weinberg et al., 2010) and eukaryotes (Torarinsson et al., 2008b), as well as in our previous discovery of the TRIT element (Gardner et al., 2011). Applying this algorithm to the filtered sequences for each genome resulted in a total of 22310 motif predictions. I searched these CMs back over the genome they were predicted from and removed from consideration motifs with very low (<100) or very high (> 3000) copy number to remove motifs with low explanatory power and non-specific motifs, respectively, or were not enriched with respect to gene terminal regions, leaving a set of 4359 putative termination motifs, approximately 2.5 per organism.

To reduce the complexity of this data set, I developed a method for clustering CMs. Two previous approaches to comparing CMs have been described in the literature. The first, known as CMcompare (Höner zu Siederdissen et al., 2010), computes the score of a so-called 'link sequence', that is a sequence with the highest value of $\min(S_1(s), S_2(s))$, where $S_x(s)$ is the score of sequence s with respect to model S_x. While this has been proposed as a measure of CM specificity in the context of the Rfam database, it is unclear how accurately this single link sequence captures the overlap between the sequence spaces described by two CMs, let alone the reality of overlaps in actual biological sequence databases. A second method, proposed as part of the Evofam pipeline for automated ncRNA family discovery in eukaryotic genome alignments (Parker et al., 2011), approximates the Kullback-Leibler divergence between two CMs, that is the (dis)similarity of the probability distributions over sequences emitted by the two CMs, using the difference in Infernal CM E-value calculations on a human reference sequence from each model's training set. In the context of the Evofam pipeline, the use of the human reference sequence is justifiable, as the study was primarily concerned with the discovery of ncRNA families present in the human genome. However, in the present case of clustering motifs across an entire domain of life, there is no obvious single sequence to use as a reference for the purposes of a comparison between every pair of CMs.

I have developed a sampling based approach to measuring CM similarity, inspired by discussions of using summed bitscores as a measure of remote homology between CMs (personal communication, Paul P. Gardner and Sean R. Eddy) and the reciprocal BLAST measure used by TRIBE-MCL (Enright et al., 2002). Rather than using a single

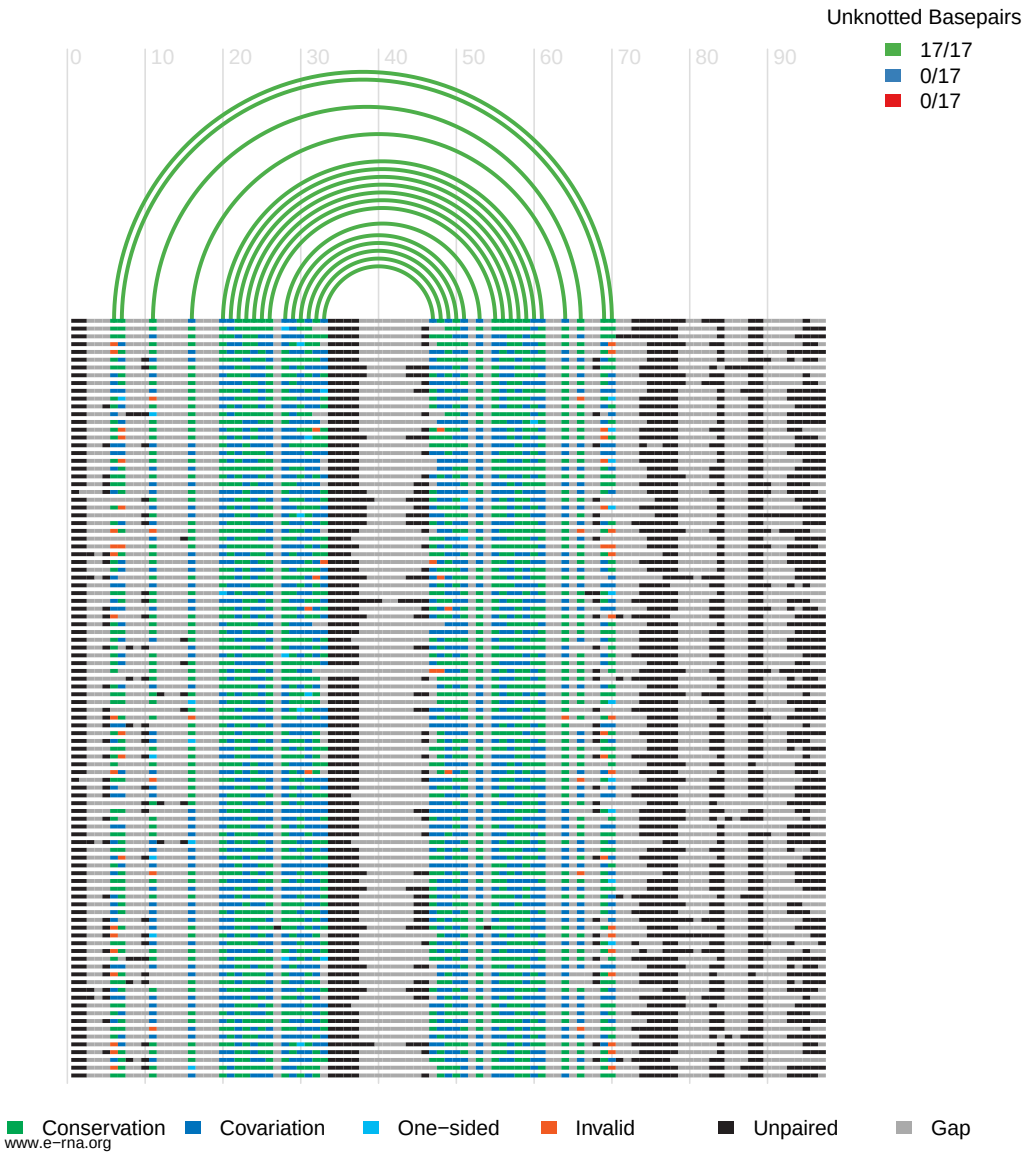


Figure 5.1: Example alignment of cluster consensus sequences. Partial alignment of the consensus sequences for cluster 16, visualized using the R-CHIE webserver (Lai et al., 2012). Green arcs represent base-pairing interactions. Nucleotides are visualized as blocks below, and are colored to highlight conservation and covariation in base-pairing relationships within the stem-loop structure.

reference sequence for the purpose of comparison, I use the fact that CMs are generative models to measure the average similarity of of their respective sequence spaces. Infernal reports bitscores and E-value for each match between a CM and a given sequence region. The bitscore, ignoring the specifics of algorithm used (either CYK or Inside), is

$$S = \log_2 \left(\frac{P(x \mid H)}{P(x \mid R)} \right)$$

where $P(x \mid H)$ is the probability of sequence x under model H, and $P(x \mid R)$ is the probability of x under a null model R, generally an iid sites model with a geometric length distribution. This score is expected to follow a Type 1 Extreme Value (or Gumbel) distribution (Karlin et al., 1990; Eddy, 2008), and this empirically appears to be the case for Infernal scores (Nawrocki et al., 2007). Hence the E-value can be calculated as

$$e^{-\lambda(S-\mu)}$$

where λ and μ are fitted parameters depending on the size of the database searched and the model architecture, and normalize for these factors. So the reciprocal similarity score (RSS) I have defined:

$$RSS_{x,y} = \left[\frac{\sum_{i=1}^{n} -\ln\left(E_{x,y,i}\right) + \sum_{j=1}^{n} -\ln\left(E_{y,x,j}\right)}{2n} \right] + \ln(n)$$

where $E_{x,y,i}$ is the E-value of the ith sequence emitted by model x scored by model y, can be understood as the average normalized bitscore of each model over the other's sequence space, and is similar in spirit to Monte Carlo approximations to the Kullback-Leibler divergence (Parker et al., 2011; Juang et al., 1985).

This measure appeared robust to the number of samples used, but this may depend in part on model complexity. As the maximal E-value in this case is n, $-\ln(n)$ is a theoretical lower bound on the average $-\ln(E)$, and the subtraction of this factor ensures that the RSS is strictly positive. It is worth noting that this measure is symmetric ignoring sampling error. Asymmetric variants may have some applications. For instance, by taking the minimum of the average bitscore under either model, one would give preference to full-length model matches in comparisons between models of various sizes due to the glocal nature of Infernal search (global with respect to the model, local with

respect to sequence), and this may be preferable for determining similarity between ncRNA families. Conversely, taking the maximum may have some utility in searching for shorter motifs. In the current application, I expect all CMs to be of roughly similar sizes and symmetric measures simplify clustering. This measure should be applicable to any generative model, and so could be similarly used to cluster e.g. HMMs.

A related measure was previously used by the TRIBE-MCL algorithm to cluster protein families based on reciprocal $\log_{10}$ BLAST E-values (Enright et al., 2002). The MCL algorithm is described in detail elsewhere (Van Dongen, 2008), but in brief it uses simulations of random walks on a weighted graph to define clusters through an unsupervised, iterative process. Unsurprisingly, many of the clusters that were generated using MCL with RSSes appeared to be composed of CMs representing canonical RITs on visual inspection with some notable exceptions, described below. However, despite a complete lack of phylogenetic assumptions in our pipeline, we found that the majority of clusters were dominated by one or two orders, generally within the same phyla, and sometimes even a single genera. This both validates our clustering procedure and indicates that RITs, despite their small size and stereotypical sequence composition, carry a phylogenetic signal when considered in aggregate.

To further study lineage-specific biases in terminator composition, I took the top 100 clusters, ranging in size from 332 to 6 CMs, and constructed consensus models through a semi-automated process. First, for each cluster I selected the 10 CMs with the highest sum of RSS scores with other cluster CMs (or all CMs in the case of clusters with < 10 members), and searched these across all of the genomes the cluster CMs were derived from. Regions that these CMs agreed were likely to be terminator sequences were collected and aligned using MAFFT Q-INS-i (Katoh et al., 2008), a heuristic Sankoff alignment algorithm which considers both sequence and secondary structure in alignment, and secondary structure was predicted using CentroidAlifold (Hamada et al., 2009), and manually refined using RALEE (Griffiths-Jones, 2005) (see figure 5.1; see also Methods for detailed alignment protocol). I annotated the 1853 EMBL files we started with, and iteratively removed from consideration any model with at least 85% of its sequence hits covered by another model. This left 16 putative terminator models, on which all further analysis was done.

5.3.2 Canonical RIT diversity

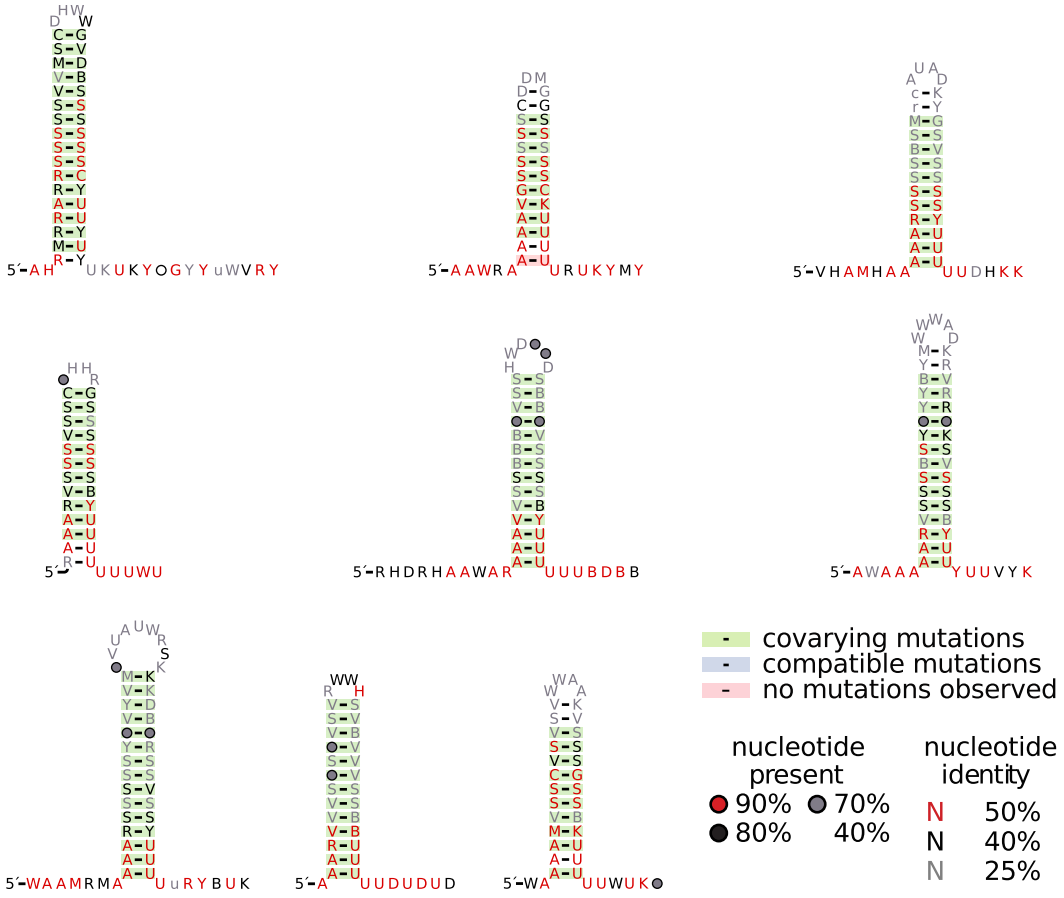


Figure 5.2: Most informative sequence for nine canonical RIT clusters. Each cluster consensus model was searched across all genomes and sequence hits with an expected FDR of 1% were aligned to the model. Duplicate sequences were removed and 5000 randomly sampled sequences were used to calculate the most informative sequence (MIS), a projection of any bases with frequencies above .25 onto IUPAC characters (Freyhult et al., 2005). Structures were drawn using R2R (Weinberg et al., 2011). From left to right, images shown represent consensus alignments for clusters 16, 18, 25 (top row); 29, 37, 88 (middle row); 89, 95, and 96 (bottom row).

Of the 16 resulting clusters, 9 appeared to be canonical RITs on visual inspection (see figure 5.2). All shared known features of canonical RITs, including a 5′ poly-A region, a G/C-rich hairpin, and a poly-U tail, but differ in stem length, hairpin loop length, and base composition. An interesting feature of these models is the universal

presence of base-pairing interactions between the poly-A and poly-U regions. Though it has been widely assumed that the poly-A region's function is primarily to contribute to bidirectional activity of RITs, some studies have shown that complementarity between the poly-A and poly-U region increase termination efficiency (Abe et al., 1996; Chen et al., 2013), presumably by contributing to the ratcheting effect of hairpin formation on the poly-U tail. In fact, a recent study showed that strong terminators with clear poly-A regions generally do not posses strong bidirectional activity, suggesting that the primary function of the poly-A region is to contribute to this ratcheting (Chen et al., 2013). I have observed covariation within many of the A-U pairs in our terminator models, supporting this observation.

5.3.2.1 Validating RIT activity with RNA-seq

To validate RIT predictions, publicly available RNA-seq datasets were collected and plots summarizing the behavior of transcription across the predictions were created (see Methods for details). There are some difficulties in using RNA-seq data to validate terminator activity. In perfect digital transcriptomic data, we would expect to observe the majority of transcripts terminating precisely within the poly-U tail of annotated RITs. Unfortunately, modern high-throughput sequencing technologies do not sequence complete RNA molecules, rather sequencing short stretches of size-selected fragmented RNA libraries. These fragments in these libraries are incidentally selected for sequence composition during both library amplification through PCR and sequencing, often with poorly understood biases, giving rise to the characteristically hilly appearance of these data sets when visualized. Additonally, protocols for the sequencing of RNA retaining strand information generally sequence all fragments of a particular RNA molecule in the same direction (for example, see Croucher et al. (2009)). As a result, if we assume that the fragmentation proceeds roughly by a Poisson process, this will naturally lead to an exponential decay in apparent expression along the $3'$ region of each transcript. Newer data sets with longer read lengths tend to give cleaner indications of termination activity. Finally, in some data sets we observed patterns of reported transcription that are suggestive of degradation of the RNA by $3'$ exonucleases or high levels of genomic DNA contamination.

Despite these potential problems, when taken in aggregate, a clear signal from the

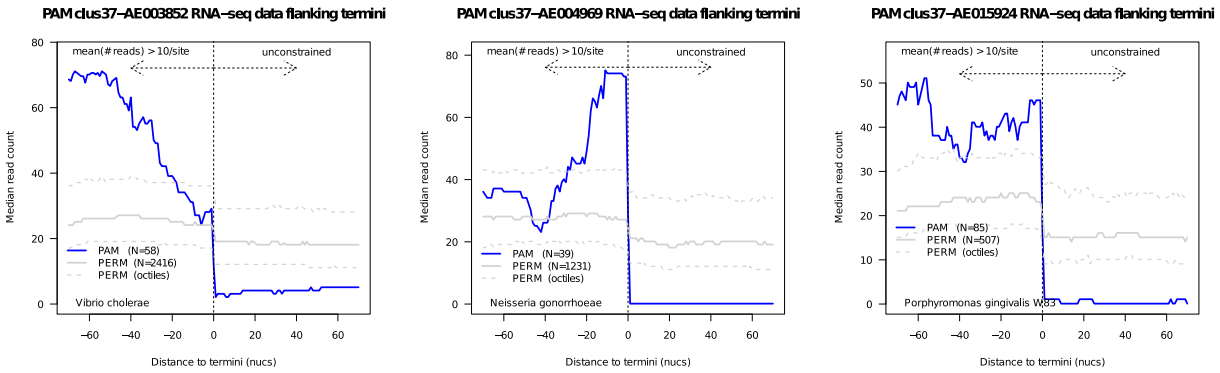


Figure 5.3: Analysis of diverse RNA-seq datasets confirm canonical terminator activity. These plots present representative analysis for terminus associated motifs (TAMs) predicted by the cluster 37 canonical terminator consensus model. The median expression over TAMs with an upstream mean expression of at least 10 reads per position is plotted in blue. Random positions meeting this same constraint are plotted in grey, and the dashed grey lines provide a 75% confidence interval for this estimate. RNA-seq data (from left to right) drawn from experiments in the γ-proteobacterium *Vibrio cholerae* (Mandlik et al., 2011), the β-proteobacterium *Neisseria gonorrhoeae* (Isabella et al., 2011), and the Bacteroidetes *Porphyromonas gingivalis* (Høvik et al., 2012).

termination activity of canonical RITs can be observed (see figure 5.3 for examples). These plots present median read counts over predicted RITs as a robust estimator of the mean expression. As a control, the median read counts over randomly selected positions were similarly selected. A clear difference in the change in the level of transcription over RITs can be observed as compared to random positions, often much larger than the difference between the top estimate of a 75% confidence interval before and the bottom estimate after these randomly positions. This pattern appears to hold for all of the canonical RIT clusters discovered in the course of this work. I did observe cases where there did not appear to be the characteristic drop in transcription across predicted canonical RITs; however, these could generally be attributed to high levels of 'background transcription' (possibly resulting from sample contamination with genomic DNA) confounding the selection criteria on element upstream transcription (see methods for details). An adaptive selection criteria based on the median absolute deviation from the median transcription across all positions in the genome, rather than an arbitrary cut-off on mean transcription, may correct this, and we are currently pursuing this possibility. As it stands, these plots provide a qualitative indication of termination activity. However,

it should be possible to quantify these results using, e.g., a permutation test on the change in median transcription over random samples of the same size as the number of predicted RITs meeting the upstream transcription selection criterion.

5.3.2.2 Lineage-specific enrichment of canonical RIT clusters

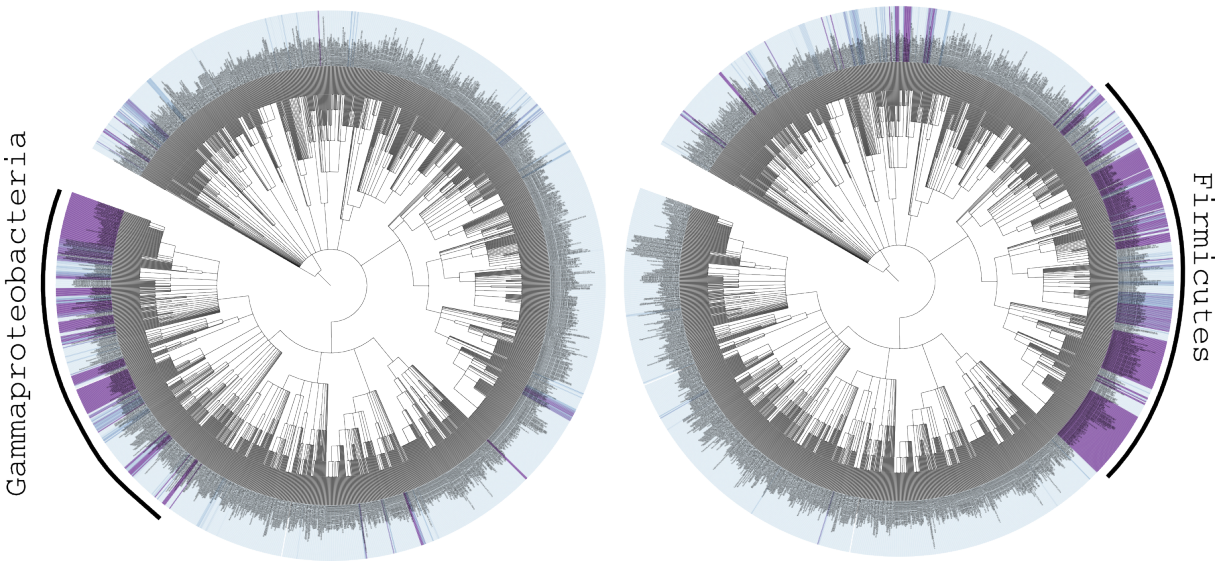


Figure 5.4: Canonical RIT enrichment on the NCBI taxonomy. These figures show the extent of canonical RIT enrichment at an FDR of 5% in each genome for canonical RIT clusters 18 (left) and 37 (right). Each leaf node represents a single genome, and colors represent -$\log_{10}$ hypergeometric p-values ranging on a scale from light blue (no enrichment) to purple (high enrichment). Clades with large numbers of enriched genomes are annotated. Figures drawn using the Interactive Tree of Life webserver (Letunic et al., 2011).

As noted previously, many of the clusters recovered by the motif-discovery pipeline appeared to consist largely of elements discovered in related genomes. The final consensus alignments constructed from these clusters have broadly similar architectures (see figure 5.2), so it was unclear if they would retain the characteristics which allowed the RSS-based MCL clustering to recover the phylogenetic relationships between host genomes. To provide an initial assessment of the lineage-specificity of the motifs, I performed a hypergeometric test for element enrichment in each genome for each cluster. This revealed clear patterns of lineage-specific enrichment for each element (see figure 5.4 for

representative examples).

Two alternative hypotheses could explain these patterns. The first, which I will term the global selection hypothesis, is selection for a particular form of terminator motif. This could be either active selection for robust terminator activity in the face of an evolving transcription apparatus (Iyer et al., 2004), or an incidental effect of selection for other genomic properties such as G/C content, or more likely, a combination of both. The second, which I will call the transposition hypothesis, would be based on the distribution of particular RIT forms by transposable elements and would imply an evolutionary relationship between members of a particular RIT cluster. Transposable elements have previously been suggested as a means for the distribution of RITs later exapted as elements of 5′ cis-regulatory elements by Naville et al. (2010), and there is no reason a similar mechanism could not deposit 3′ RITs. Given the apparently ancient origins of many of the observed lineage-specific enrichments, the deposited RITs would subsequently have to be somewhat protected from random mutations preserving termination activity by a selective process, though as the degree of sequence and structural divergence allowed by the CM-based classification is currently unclear, this may well be possible. Of course, these two hypotheses are not mutually exclusive, and could act together to explain the observed pattern of terminator enrichment. It is important to note that enrichment of one RIT cluster does not imply the exclusion of alternative terminator structures in a particular genome. As seen in figure 5.3 RIT clusters are present and apparently active outside the genomes they are enriched in; in this case, cluster 37, enriched primarily in the Firmicutes, is present at fairly low copy numbers in other phyla. Whether this reflects convergence or shared descent of these elements is unclear.

5.3.3 Non-canonical putative attenuation motifs

Besides the canonical RITs discussed so far, the motif discovery pipeline uncovered 7 clusters which did not fit the canonical RIT model of a G/C-rich hairpin followed by a poly-U tract. I will refer to these elements as terminus associated motifs (TAMs). These elements tend to have much narrower host ranges than the canonical RITs discussed above; I discuss a few of them in the following sections.

5.3.3.1 The Neisserial DNA uptake sequence TAM

This first, and perhaps one of the most distinctive, of these elements is a previously known TAM containing a DNA uptake sequence (DUS) in the β-proteobacterial order Neisseriales. The Neisseriales frequently exchange genetic material, leading to difficulties in studying their population structure and so-called 'fuzzy' species (Corander et al., 2012). This exchange is mediated by specific systems (Hamilton et al., 2006). Neisserial species are able to excrete DNA for donation through a type 4 secretion system (Hamilton et al., 2005) and/or autolysis. A type 4 pilus-like system is then thought to specifically bind DNA containing a 10-base DUS (GCCGTCTGAA in *Neisseria gonorrhoeae*), which is then incorporated in to the genome through homologous recombination. Recent work has shown that there are a number of distinct 'dialects' of DUS which act to reduce the efficiency of uptake between distantly related species within the order (Frye et al., 2013).

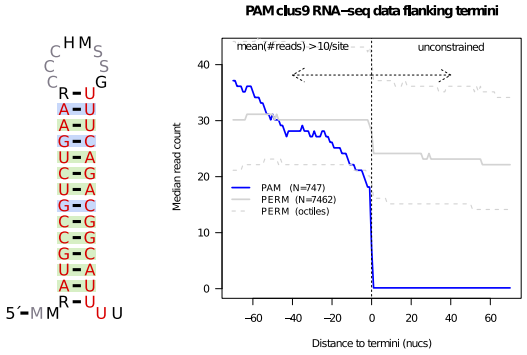


Figure 5.5: Neisserial DNA uptake sequence terminator. On the left, consensus secondary structure and MIS for 2012 non-identical cluster 9 TAMs in the order Neisseriales. On the right, median expression over predicted terminator sequences derived from RNA-seq experiments in *Neisseria gonorrhoeae* (Isabella et al., 2011).

The presence of the *Neisseria* DUS in terminator-like structures has long been noted (Goodman et al., 1988), and was discussed extensively in the study reporting the development of TransTermHP (Kingsford et al., 2007). However, the termination activity of this element has never been experimentally tested. Using our RNA-seq collection, we are able to show that this element is indeed associated with a sharp drop in transcription (see figure 5.5).

5.3.3.2 The Actinobacterial TAM

The motif discovery pipeline also recovered a motif redundant with the one previously dubbed TRIT in chapter 4 and Gardner et al. (2011), cluster 7. The enrichment analysis I performed indicated that rather than being restricted to the Mycobacteria as we previously hypothesized, this element appears to occur throughout the Actinobacteria.

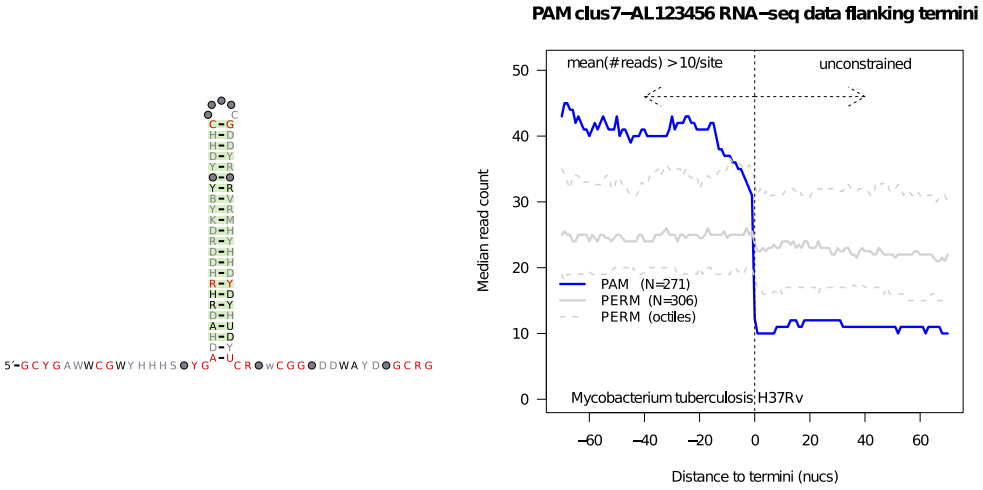


Figure 5.6: Actinobacterial TAM. On the left, consensus secondary structure and MIS for 2891 non-identical cluster 7 TAMs in the class Actinobacteria. On the right, median expression over predicted terminator sequences derived from RNA-seq experiments in *Mycobacterium tuberculosis* (Arnvig et al., 2011).

This motif also overlaps with two 'I-shaped' elements previously discovered in an MFE-based screen for non-canonical termination motifs (Unniraman et al., 2001), downstream of the *Mycobacterium tuberculosis* genes *tuf* and *Rv1324*. These structures have previously been shown to reduce expression of downstream genes by ∼80% in synthetic constructs *in vivo* in *Mycobacterium smegmatis*, and to specifically terminate transcription *in vitro*. The results of our RNA-seq analysis (see figure 5.6) suggest that this termination activity holds for the entire class of these elements.

Interestingly, the enrichment analysis also showed overrepresentation of hits in a number of Proteobacterial genera, including *Pseudomonas* species. Analysis of RNA-seq data in *Pseudomonas putida* (Frank et al., 2011), which harbors ∼300 putative copies of this element, showed no evidence of involvement in transcription termination. An alignment generated from the putative *Pseudomonas* sequences contained extended G/C-rich sequence within the loop region of the motif, which could potentially form an extended secondary structure. Together, this suggests that the *Pseudomonas* element is not a member of the same class as the Actinobacterial element, and these hits may be a result of low specificity in the cluster consensus model, likely due to partial similarity

between the stem structure of the two elements confounding the RSS measure. A second cluster with exclusively Actinobacterial sequences was also discovered by MCL, and it is possible this has higher specificity for the TAM. Alternatively, the specificity of the cluster 7 model could potentially be increased by removing non-Actinobacterial seqeunces from the alignment.

5.3.3.3 Type 1 integron attC sites

Many Gram-negative bacteria harbor arrays of horizontally-acquired gene cassettes known as integrons (Hall, 2012). The architecture of these integrons is roughly similar, consisting of an *intI* gene encoding an integrase, an *attI* integration site, and a series of gene cassettes containing *attC* sites important for recognition by IntI. While the sequence of *attC* sites can vary widely, it has long been known that the *attC* sites of the *Vibrio cholerae* type 1 integron are unusually homogenous. My pipeline discovered this motif (see figure 5.7), and it is enriched primarily in *Vibrio* and *Shewanella* genomes, though can be found sporadically at low copy number throughout the γ-proteobacteria. Expression of type 1 integrons is thought to be driven primarily by a single upstream promoter. An early study of this expression suggested that the *attC* sites may be acting as transcriptional terminators based on Northern blots showing that transcripts did not cover the entire integron and tended to contain full-length gene cassettes, and that transcript frequency was inversely correlated with transcript length (Collis et al., 1995). A single study has attempted verify this hypothesis, and found that *attC* sites do not appear to promote transcriptional termination, and rather propose a mechanism for enhancing cassette expression through the presence of short ORFs within the *attC* sites (Jacquier et al., 2009). However, this study only tested a single *attC* site with an atypically large hairpin-loop region for termination

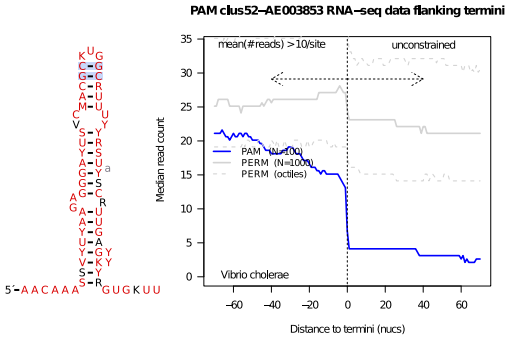


Figure 5.7: Type 1 integron attC sites. On the left, consensus secondary structure and MIS for 420 cluster 52 TAMs in the Proteobacteria. On the right, median expression over predicted terminator sequences derived from RNA-seq experiments in *Vibrio cholerae* (Mandlik et al., 2011).

activity; additionally this study does not explain the patterns seen in the Northern blots of the Collis et al. (1995) study. A recent study of the termination efficiency of a large number of transcriptional terminators included an *attC* site in their initial screens, though it was discarded early in their study as being a low efficiency terminator (Cambray et al., 2013). However, their initial experiments on this element, using a fluorescent reporter construct in *Escherichia coli*, did show a termination efficiency of 25%. Our analysis of RNA-seq data in *Vibrio cholerae* appears to support the hypothesis that at least some *attC* may operate as transcriptional attenuators. This stochastic attenuation at *attC* sites would explain the results of Collis et al. (1995), and would lead to a gradual titration of expression along the length of integrons, barring the presence of internal promoters.

5.3.3.4 Other non-canonical TAMs

Four other non-canonical TAMs were identified by the motif-discovery pipeline. One of these appears to be a simple repeat family in the β-proteobacteria, and RNA-seq analysis indicates it is likely not involved in transcriptional termination. I am still investigating the potential activity of the other three at the time of writing.

5.4 Discussion

In a recent comprehensive review of transcriptional termination, Peters et al. (2011) lay out four criteria for experimental validation of transcriptional terminators:

> 1) it causes dissociation of (the elongation complex) during *in vitro* transcription as detected by release of RNA and DNA from RNAP; 2) it generates terminated RNA 3′-ends before readthrough transcripts appear during synchronized *in vitro* transcription; 3) it generates the terminated RNA 3′-ends *in vivo*; and 4) it significantly reduces synthesis of RNA downstream from the site *in vivo*.

A primarily computational study as described here cannot hope to meet this burden of evidence. Indeed, the authors of this review admit that only a small number of even canonical RITs have been subjected to this degree of validation, and furthermore discuss a number of cases where even "obvious" RITs have turned out not to function as

transcriptional terminators. However, while I can not rule out with certainty alternative explanations for the transcriptional patterns I have observed over predicted TAMs, such as protection from 3′ exonucleases, I believe that the evidence I have presented here in combination with previous studies suggesting possible non-canonical termination motifs is indicative of a wider diversity of intrinsic termination mechanisms than is immediately evident from studies in model organisms.

While the work presented here provides initial insights into the diversity of elements associated with transcriptional termination, there remain a number of issues that need to be addressed in this study. Foremost is the criterion used to define the set of TAMs which I carried forward for enrichment and transcriptional analysis, that is <85% overlap with all other TAMs across the phylogeny. It is well known that currently available genome sequences are highly biased towards a relatively small number of organisms that are easily cultivated; furthermore, this set is itself biased towards model species and human pathogens, which may not be representative of the phylogeny as a whole. It is possible that a more nuanced criterion, based for instance on overlaps at the class level, may provide a clearer picture of terminator diversity. Providing this view of terminator diversity will be increasingly important as our understanding of bacterial diversity expands in light of sequencing projects targeting underrepresented genera (Wu et al., 2009) and the difficult to cultivate 'dark matter' of the phylogeny through single-cell sequencing (Marcy et al., 2007; Rinke et al., 2013).

A second major challenge to be addressed is identifying the determinants which allow the CMs I have constructed to distinguish between classes of RITs in various lineages. These determinants may include the sequence compositions of particular regions or base-pairs within the terminator structure, or gross aspects of each class such as stem-length and G/C content. It is well known that the specific sequence composition of RITs can have large effects on termination efficiency in *Escherichia coli*, even when maintaining the canonical G/C-rich hairpin followed by a poly-U tail (Chen et al., 2013; Cambray et al., 2013). It seems likely that evolution of the transcriptional apparatus would change these design constraints, and I believe the methods I have developed in this study may allow us to begin to probe the parameters which may underlie RIT function in diverse host species.

Publications

Publications arising in the course of this thesis:

- Read H., Johnson S., Barquist L., Mills G., Gardner P.P., Patrick W.M., Wiles S. **The effect of constitutive bioluminescence expression on the in vitro and in vivo fitness of the mouse enteropathogen *Citrobacter rodentium*.** Manuscript in preparation.

- Okoro C.K., Barquist L., Kingsley R.A., Connor T.R., Harris S.R., Arends M., Stevens M., Parry C.M., Al-Mashhadani M.N., Kariuki S., Msefula C.L., Gordon M.A., de Pinna E., Wain J., Heyderman R.S., Obaro S., Alonso P.L., Mandomando I., MacLennan C.A., Tapia M.D., Levine M.M., Tennant S.M., Parkhill J., Dougan G. **Signatures of adaptation in human invasive *S.* Typhimurium populations.** Manuscript in preparation.

- Wong V., Pickard D., Barquist L., Sivaraman K., Hart P., Arends M., Holt K., Kane L., Mottram L., Ellison L., Kay S., Wileman T., Kenney L., MacLennan C., Kingsley R.A., Dougan G. **Characterization of the yehUT two-component regulatory system of *Salmonella enterica* serovars Typhi and Typhimurium**. Manuscript under review.

- Wilf N.M., Reid A.J., Ramsay J.P., Williamson N.R., Croucher N.J., Gatto L., Hester S.S., Goulding D., Barquist L., Lilley K.S., Kingsley R.A., Dougan G., Salmond G.P.C.. **RNA-seq reveals the RNA binding proteins, Hfq and RsmA, play various roles in virulence, antibiotic production and genomic flux in *Serratia* sp. 39006**. Manuscript under review.

- Pettit L.J., Browne H.P., Yu L., Smits W.K., Fagan R.P., Barquist L., Martin M.J., Goulding D., Duncan S.H., Flint H.J., Dougan G., Choudhary J.S., Lawley T.D. **Functional genomics reveals that *Clostridium difficile* Spo0A coordinates sporulation, virulence and metabolism.** Manuscript under review.

- Reuter S., Connor T.R., Barquist L., Walker D., Feltwell T., Harris S.R., Fookes M., Hall M.E., Fuchs T.M., Corander J., Dufour M., Ringwood T., Savin C., Bouchier C., Martin L., Miettinen M., Shubin M., Laukkanen-Ninios R., Sihvonen L.M., Siitonen A., Skurnik M., Falcão J.P., Fukushima H., Scholz H.C., Prentice M., Wren B.W., Parkhill J., Carniel E., Achtman M., McNally A., Thomson N.R. **Parallel independent evolution of pathogenicity within the genus *Yersinia*.** Manuscript under review.

- Barquist L., Burge S.W., Gardner P.P. **Building non-coding RNA families.** *Methods in Molecular Biology*, in press.

- Hoeppner M.P., Barquist L., Gardner P.P. **An introduction to RNA databases.** *Methods in Molecular Biology*, in press.

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Appendix A: Supplementary data for chapters 2 and 3

This thesis should include a CD containing supplementary data for chapters 2 and 3. This CD contains two files in Excel format.

Chapter2.xls contains the complete results of the TraDIS assays described in chapter 2, and should be identical to the supplementary information of Barquist et al. (2013b).

Chapter3.xls contains genomic features significantly depleted or enriched in insertions over the macrophage assays described in chapter 3.

If the CD is not enclosed, or if you are viewing this thesis electronically, contact Lars Barquist (lb14@sanger.ac.uk) to obtain these files.

Appendix B: Genomic sequences analyzed for termination motifs

Genomic sequences analyzed for termination motifs in chapter 5

EMBL accession	Scientific name
AP011945	Helicobacter pylori F57
CP000885	Clostridium phytofermentans ISDg
CP000471	Magnetococcus marinus MC-1
BA000033	Staphylococcus aureus subsp. aureus MW2
CP000679	Caldicellulosiruptor saccharolyticus DSM 8903
CP000049	Borrelia turicatae 91E135
CP002534	Cellulophaga lytica DSM 7489
CP002876	Nitrosomonas sp. Is79A3
AM286280	Francisella tularensis subsp. tularensis FSC198
CP002505	Rahnella sp. Y9602
AE016958	Mycobacterium avium subsp. paratuberculosis K-10
CP000950	Yersinia pseudotuberculosis YPIII
FR856862	Novosphingobium sp. PP1Y
CP002819	Ralstonia solanacearum Po82
FP929043	Eubacterium rectale M104/1
CP002621	Enterococcus faecalis OG1RF
FP929034	Bifidobacterium longum subsp. longum F8
CP001150	Rhodobacter sphaeroides KD131
CP002302	Buchnera aphidicola str. JF99 (Acyrthosiphon pisum)
CP001158	Buchnera aphidicola str. Tuc7 (Acyrthosiphon pisum)
AP012205	Synechocystis sp. PCC 6803
AE015927	Clostridium tetani E88
CU469464	Candidatus Phytoplasma mali
CP001032	Opitutus terrae PB90-1
CP002805	Chlamydophila psittaci 01DC11
CP000946	Escherichia coli ATCC 8739
CP000529	Polaromonas naphthalenivorans CJ2
CP001071	Akkermansia muciniphila ATCC BAA-835
CP001336	Desulfitobacterium hafniense DCB-2
AE017126	Prochlorococcus marinus subsp. marinus str. CCMP1375
CP002218	Burkholderia sp. CCGE1003
BX571857	Staphylococcus aureus subsp. aureus MSSA476
AP011149	Acetobacter pasteurianus IFO 3283-26
AE005174	Escherichia coli O157
AM422018	Candidatus Phytoplasma australiense
CP001581	Clostridium botulinum A2 str. Kyoto
AE017283	Propionibacterium acnes KPA171202
CP002810	Isoptericola variabilis 225
CP000813	Bacillus pumilus SAFR-032
CP001752	Treponema pallidum subsp. pallidum str. Chicago

CP000348	Leptospira borgpetersenii serovar Hardjo-bovis str. L550
BX571963	Rhodopseudomonas palustris CGA009
CP002811	Shewanella baltica OS117
AE013598	Xanthomonas oryzae pv. oryzae KACC 10331
CP002881	Pseudomonas stutzeri ATCC 17588 = LMG 11199
DS180873	Leptospirillum rubarum
CP002829	Thermodesulfobacterium sp. OPB45
CP000606	Shewanella loihica PV-4
AM902716	Bordetella petrii
CP000076	Pseudomonas protegens Pf-5
AP009389	Pelotomaculum thermopropionicum SI
CP001037	Nostoc punctiforme PCC 73102
CU468135	Erwinia tasmaniensis Et1/99
CP002026	Starkeya novella DSM 506
FP929051	Ruminococcus bromii L2-63
CP000924	Thermoanaerobacter pseudethanolicus ATCC 33223
CP000553	Prochlorococcus marinus str. NATL1A
CP002728	Tepidanaerobacter acetatoxydans Re1
CP002312	Borrelia burgdorferi JD1
CP000384	Mycobacterium sp. MCS
CP002521	Acidovorax avenae subsp. avenae ATCC 19860
FN433596	Staphylococcus aureus subsp. aureus TW20
CM000728	Bacillus cereus Rock1-3
CR522870	Desulfotalea psychrophila LSv54
CP001509	Escherichia coli BL21(DE3)
AB097150	Onion yellows phytoplasma
CP001960	Campylobacter jejuni subsp. jejuni S3
CP000943	Methylobacterium sp. 4-46
CP000667	Salinispora tropica CNB-440
CP001958	Segniliparus rotundus DSM 44985
CM000604	Clostridium difficile ATCC 43255
CP002660	Clostridium acetobutylicum DSM 1731
CP001794	Geobacillus sp. Y412MC61
CP002868	Spirochaeta caldaria DSM 7334
CP001959	Brachyspira murdochii DSM 12563
FM242711	Listeria monocytogenes serotype 4b str. CLIP 80459
FM204884	Streptococcus equi subsp. zooepidemicus
CP002442	Geobacillus sp. Y412MC52
CP000680	Pseudomonas mendocina ymp
CP002777	Thermus thermophilus SG0.5JP17-16
FN568063	Streptococcus mitis B6
CP000386	Rubrobacter xylanophilus DSM 9941
FR668087	Mycoplasma leachii 99/014/6
AP010888	Bifidobacterium longum subsp. longum JCM 1217
CP001124	Geobacter bemidjiensis Bem
AM040264	Brucella melitensis biovar Abortus 2308
CP001801	Halothiobacillus neapolitanus c2
CP002416	Clostridium thermocellum DSM 1313
CP000458	Burkholderia cenocepacia HI2424
CP000828	Acaryochloris marina MBIC11017
CP002890	Escherichia coli UMNF18
BA000045	Gloeobacter violaceus PCC 7421
AP006628	Onion yellows phytoplasma OY-M
CP001981	Candidatus Sulcia muelleri DMIN
CP000932	Campylobacter lari RM2100
CP000774	Parvibaculum lavamentivorans DS-1
CP001903	Bacillus thuringiensis BMB171
CP002164	Caldicellulosiruptor obsidiansis OB47
CP002825	Lacinutrix sp. 5H-3-7-4
CP001196	Oligotropha carboxidovorans OM5
CP000869	Burkholderia multivorans ATCC 17616
AP012030	Escherichia coli DH1
CP000837	Streptococcus suis GZ1
CP001488	Brucella melitensis ATCC 23457
CP002542	Fluviicola taffensis DSM 16823
CP000685	Flavobacterium johnsoniae UW101
CP001251	Dictyoglomus turgidum DSM 6724

CP002767	Shewanella baltica BA175
CP002334	Helicobacter pylori Lithuania75
CP001184	Ureaplasma urealyticum serovar 10 str. ATCC 33699
CP001930	Chlamydia trachomatis G/9301
BA000008	Chlamydophila pneumoniae J138
CP000803	Francisella tularensis subsp. holarctica FTNF002-00
AP009484	Macrococcus caseolyticus JCSC5402
CP002593	Pseudonocardia dioxanivorans CB1190
CP001504	Burkholderia glumae BGR1
CM000738	Bacillus cereus AH676
CP000248	Novosphingobium aromaticivorans DSM 12444
AE017194	Bacillus cereus ATCC 10987
CP000705	Lactobacillus reuteri DSM 20016
CP001281	Thauera sp. MZ1T
FP236530	Mycoplasma hominis ATCC 23114
CP000154	Paenibacillus polymyxa E681
CP000283	Rhodopseudomonas palustris BisB5
CM000776	Helicobacter canadensis MIT 98-5491
AM233362	Francisella tularensis subsp. holarctica LVS
CP001100	Chloroherpeton thalassium ATCC 35110
AE017196	Wolbachia endosymbiont of Drosophila melanogaster
BX897700	Bartonella quintana str. Toulouse
CM000747	Bacillus thuringiensis Bt407
CP002120	Staphylococcus aureus subsp. aureus str. JKD6008
CP002627	Bacillus amyloliquefaciens TA208
CP002739	Thermoanaerobacterium xylanolyticum LX-11
CP002345	Paludibacter propionicigenes WB4
AP012035	Acidiphilium multivorum AIU301
CU234118	Bradyrhizobium sp. ORS 278
CP001744	Planctomyces limnophilus DSM 3776
CP000875	Herpetosiphon aurantiacus DSM 785
CP002300	Buchnera aphidicola str. LL01 (Acyrthosiphon pisum)
AM990992	Staphylococcus aureus subsp. aureus ST398
CP001814	Streptosporangium roseum DSM 43021
CP002526	Glaciecola sp. 4H-3-7+YE-5
CP000910	Renibacterium salmoninarum ATCC 33209
CP002927	Bacillus amyloliquefaciens XH7
CP002361	Oceanithermus profundus DSM 14977
CP002339	Alteromonas sp. SN2
CM000661	Clostridium difficile QCD-76w55
CP000108	Chlorobium chlorochromatii CaD3
CP002468	Bacillus subtilis BSn5
CP002096	Helicobacter pylori 35A
CP002080	Acinetobacter oleivorans DR1
CR628337	Legionella pneumophila str. Lens
CP002124	Erwinia sp. Ejp617
CP000688	Dehalococcoides sp. BAV1
AE016795	Vibrio vulnificus CMCP6
CP000026	Salmonella enterica subsp. enterica serovar Paratyphi A str. ATCC 9150
CP000030	Anaplasma marginale str. St. Maries
CP001661	Geobacter sp. M21
CP001643	Brachybacterium faecium DSM 4810
CP000675	Legionella pneumophila str. Corby
AP008957	Rhodococcus erythropolis PR4
CR354532	Photobacterium profundum SS9
CM000757	Bacillus thuringiensis serovar pulsiensis BGSC 4CC1
CM000748	Bacillus thuringiensis serovar thuringiensis str. T01001
CP002219	Caldicellulosiruptor hydrothermalis 108
CP000517	Lactobacillus helveticus DPC 4571
AP010656	Candidatus Azobacteroides pseudotrichonymphae genomovar. CFP2
CP002901	Sulfobacillus acidophilus TPY
CP000613	Rhodospirillum centenum SW
CP000115	Nitrobacter winogradskyi Nb-255
BA000012	Mesorhizobium loti MAFF303099
CP000048	Borrelia hermsii DAH
CU459003	Magnetospirillum gryphiswaldense MSR-1
CP001734	Desulfohalobium retbaense DSM 5692

CP000246	Clostridium perfringens ATCC 13124
CP002536	Deinococcus proteolyticus MRP
AE008691	Thermoanaerobacter tengcongensis MB4
AE004439	Pasteurella multocida subsp. multocida str. Pm70
CP002188	Mycoplasma bovis PG45
CP002185	Escherichia coli W
CP000847	Rickettsia akari str. Hartford
CP001598	Bacillus anthracis str. A0248
CP000023	Streptococcus thermophilus LMG 18311
FR773153	Mycoplasma haemofelis str. Langford 1
CP002110	Staphylococcus aureus subsp. aureus TCH60
CP000702	Thermotoga petrophila RKU-1
AJ235269	Rickettsia prowazekii str. Madrid E
CP001872	Mycoplasma gallisepticum str. R(high)
CP001674	Methylovorus glucosetrophus SIP3-4
AM398681	Flavobacterium psychrophilum JIP02/86
CP002273	Eubacterium limosum KIST612
CP001287	Cyanothece sp. PCC 8801
AE008692	Zymomonas mobilis subsp. mobilis ZM4
CP002516	Escherichia coli KO11FL
CP001848	Pirellula staleyi DSM 6068
CP000918	Streptococcus pneumoniae 70585
CP001978	Marinobacter adhaerens HP15
CP002616	Lactobacillus casei LC2W
CP000814	Campylobacter jejuni subsp. jejuni 81116
AP011540	Rothia mucilaginosa DY-18
CP000247	Escherichia coli 536
CP000709	Brucella ovis ATCC 25840
CP001182	Acinetobacter baumannii AB0057
CP002525	Mycoplasma suis str. Illinois
FP929033	Bacteroides xylanisolvens XB1A
CP002024	Chlamydia trachomatis L2c
CP002338	Lactobacillus amylovorus GRL 1112
FR775250	Salmonella enterica subsp. enterica serovar Weltevreden str. 2007-60-3289-1
AP007281	Lactobacillus reuteri JCM 1112
CP001322	Desulfatibacillum alkenivorans AK-01
CP000821	Shewanella sediminis HAW-EB3
AP012032	Pantoea ananatis AJ13355
CP001234	Vibrio cholerae M66-2
CP001230	Persephonella marina EX-H1
CP001886	Chlamydia trachomatis E/150
CP000727	Clostridium botulinum A str. Hall
CM000758	Bacillus thuringiensis IBL 200
FP565814	Salinibacter ruber M8
FN424405	Salmonella enterica subsp. enterica serovar Typhimurium str. D23580
FP929059	Eubacterium siraeum V10Sc8a
CM000723	Bacillus cereus BDRD-ST24
AE017340	Idiomarina loihiensis L2TR
CP001348	Clostridium cellulolyticum H10
CM000755	Bacillus thuringiensis serovar pondicheriensis BGSC 4BA1
CP002725	Gardnerella vaginalis HMP9231
CP000057	Haemophilus influenzae 86-028NP
FP885895	Ralstonia solanacearum CMR15
AM849034	Clavibacter michiganensis subsp. sepedonicus
CP001798	Nitrosococcus halophilus Nc4
BX293980	Mycoplasma mycoides subsp. mycoides SC str. PG1
FM211187	Streptococcus pneumoniae ATCC 700669
CP001844	Staphylococcus aureus 04-02981
CU468230	Acinetobacter baumannii
CM000736	Bacillus cereus F65185
CP000887	Brucella abortus S19
CP000395	Borrelia afzelii PKo
FP929038	Coprococcus catus GD/7
AE005674	Shigella flexneri 2a str. 301
AP010958	Escherichia coli O103
CP002171	Thermoanaerobacterium thermosaccharolyticum DSM 571
CP002780	Desulfotomaculum ruminis DSM 2154

CP002745	Collimonas fungivorans Ter331
CP000117	Anabaena variabilis ATCC 29413
CP002456	Taylorella equigenitalis MCE9
CP001850	Clostridiales genomosp. BVAB3 str. UPII9-5
CP002865	Zymomonas mobilis subsp. pomaceae ATCC 29192
CP001736	Kribbella flavida DSM 17836
CP000725	Streptococcus gordonii str. Challis substr. CH1
CP002582	Clostridium lentocellum DSM 5427
CP001793	Paenibacillus sp. Y412MC10
CP000416	Lactobacillus brevis ATCC 367
CP000036	Shigella boydii Sb227
CP002857	Corynebacterium resistens DSM 45100
CP000850	Salinispora arenicola CNS-205
CP002910	Klebsiella pneumoniae KCTC 2242
AE010300	Leptospira interrogans serovar Lai str. 56601
CP001635	Variovorax paradoxus S110
CP002281	Ilyobacter polytropus DSM 2926
CP002634	Bacillus amyloliquefaciens LL3
CP001855	Escherichia coli O83
BA000004	Bacillus halodurans C-125
CP000110	Synechococcus sp. CC9605
CP000075	Pseudomonas syringae pv. syringae B728a
CP000422	Pediococcus pentosaceus ATCC 25745
CR555306	Aromatoleum aromaticum EbN1
CP000031	Ruegeria pomeroyi DSS-3
CP002786	Amycolicicoccus subflavus DQS3-9A1
CP001633	Francisella tularensis subsp. tularensis NE061598
CP001634	Kosmotoga olearia TBF 19.5.1
CP000046	Staphylococcus aureus subsp. aureus COL
BX470249	Bordetella parapertussis 12822
CP001809	Corynebacterium pseudotuberculosis 1002
CP002251	Corynebacterium pseudotuberculosis I19
CP002189	Candidatus Blochmannia vafer str. BVAF
BA000040	Bradyrhizobium japonicum USDA 110
CP001680	Helicobacter pylori 52
CP000088	Thermobifida fusca YX
CP002205	Sulfurimonas autotrophica DSM 16294
AP008981	Orientia tsutsugamushi str. Ikeda
CT573326	Pseudomonas entomophila L48
CP000114	Streptococcus agalactiae A909
AL646052	Ralstonia solanacearum GMI1000
FN555004	Helicobacter mustelae 12198
CP001011	Xylella fastidiosa M23
CM000636	Mycobacterium kansasii ATCC 12478
AJ749949	Francisella tularensis subsp. tularensis SCHU S4
CP001389	Sinorhizobium fredii NGR234
CP000970	Escherichia coli SMS-3-5
CP000359	Deinococcus geothermalis DSM 11300
CP001104	Eubacterium eligens ATCC 27750
BX897699	Bartonella henselae str. Houston-1
CP000967	Xanthomonas oryzae pv. oryzae PXO99A
CP002213	Paenibacillus polymyxa SC2
CP001878	Bacillus pseudofirmus OF4
CP002689	Porphyromonas asaccharolytica DSM 20707
FQ312029	Streptococcus pneumoniae INV200
CP001727	Alicyclobacillus acidocaldarius subsp. acidocaldarius DSM 446
BX248353	Corynebacterium diphtheriae NCTC 13129
FP929044	Eubacterium siraeum 70/3
CP001026	Burkholderia ambifaria MC40-6
AE001439	Helicobacter pylori J99
CM000731	Bacillus cereus Rock3-29
CP001138	Salmonella enterica subsp. enterica serovar Agona str. SL483
CP000251	Anaeromyxobacter dehalogenans 2CP-C
CP000853	Alkaliphilus oremlandii OhILAs
CP002571	Helicobacter pylori 2017
CP000316	Polaromonas sp. JS666
CP001737	Nakamurella multipartita DSM 44233

CP001337	Chloroflexus aggregans DSM 9485
CP002121	Streptococcus pneumoniae AP200
CP000749	Marinomonas sp. MWYL1
CP000436	Haemophilus somnus 129PT
CP002608	Chlamydophila pecorum E58
CP000111	Prochlorococcus marinus str. MIT 9312
CP002160	Clostridium cellulovorans 743B
CP002299	Frankia sp. EuI1c
CP000140	Parabacteroides distasonis ATCC 8503
CP002422	Neisseria meningitidis M01-240355
CP002558	Francisella cf. novicida 3523
CP002162	Micromonospora aurantiaca ATCC 27029
CP001837	Staphylococcus lugdunensis HKU09-01
U00096	Escherichia coli str. K-12 substr. MG1655
CP002336	Helicobacter pylori SouthAfrica7
CP001145	Coprothermobacter proteolyticus DSM 5265
CP000777	Leptospira biflexa serovar Patoc strain 'Patoc 1 (Ames)'
CP000360	Candidatus Koribacter versatilis Ellin345
AE016877	Bacillus cereus ATCC 14579
CP001562	Bartonella grahamii as4aup
AM920689	Xanthomonas campestris pv. campestris
FR872580	Parachlamydia acanthamoebae UV-7
AP010968	Kitasatospora setae KM-6054
CP001797	Pseudoalteromonas sp. SM9913
CP000969	Thermotoga sp. RQ2
CP001843	Treponema primitia ZAS-2
CP001656	Paenibacillus sp. JDR-2
CP000720	Yersinia pseudotuberculosis IP 31758
CP001820	Veillonella parvula DSM 2008
CP001759	Anaplasma centrale str. Israel
AE007317	Streptococcus pneumoniae R6
BX072543	Tropheryma whipplei TW08/27
BX548174	Prochlorococcus marinus subsp. pastoris str. CCMP1986
FN806773	Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1
CP001841	Treponema azotonutricium ZAS-9
CP002419	Neisseria meningitidis G2136
CP001769	Spirosoma linguale DSM 74
CP000681	Shewanella putrefaciens CN-32
CP001191	Rhizobium leguminosarum bv. trifolii WSM2304
AE000511	Helicobacter pylori 26695
AE015924	Porphyromonas gingivalis W83
CP002159	Gallionella capsiferriformans ES-2
CP001217	Helicobacter pylori P12
CP002409	Propionibacterium acnes 266
AP012203	Porphyromonas gingivalis TDC60
CP002167	Escherichia coli UM146
FN667741	Xenorhabdus bovienii SS-2004
CM000744	Bacillus mycoides Rock3-17
CP002669	Mycoplasma hyorhinis MCLD
CM000913	Streptomyces clavuligerus ATCC 27064
CP000109	Thiomicrospira crunogena XCL-2
CM000724	Bacillus cereus BDRD-ST26
CP001291	Cyanothece sp. PCC 7424
CM000714	Bacillus cereus m1293
CP000975	Methylacidiphilum infernorum V4
CP001867	Geodermatophilus obscurus DSM 43160
AE002160	Chlamydia muridarum Nigg
CP002158	Fibrobacter succinogenes subsp. succinogenes S85
CP000096	Chlorobium luteolum DSM 273
CP001931	Thermocrinis albus DSM 14484
AE017334	Bacillus anthracis str. 'Ames Ancestor'
CP001650	Zunongwangia profunda SM-A87
CP000578	Rhodobacter sphaeroides ATCC 17029
CP000634	Agrobacterium vitis S4
AP008232	Sodalis glossinidius str. 'morsitans'
AM889285	Gluconacetobacter diazotrophicus PAl 5
FQ859185	Streptomyces cattleya NRRL 8057 = DSM 46488

BA000035	Corynebacterium efficiens YS-314
AP011142	Acetobacter pasteurianus IFO 3283-22
AP011941	Helicobacter pylori F30
AP011135	Acetobacter pasteurianus IFO 3283-07
CP001407	Bacillus cereus 03BB102
CP000090	Ralstonia eutropha JMP134
CP000419	Streptococcus thermophilus LMD-9
CP002086	Nitrosococcus watsonii C-113
CP002439	Staphylococcus pseudintermedius HKU10-03
AE016825	Chromobacterium violaceum ATCC 12472
FP929036	Butyrivibrio fibrisolvens 16/4
CP000253	Staphylococcus aureus subsp. aureus NCTC 8325
AE016822	Leifsonia xyli subsp. xyli str. CTCB07
CP000095	Prochlorococcus marinus str. NATL2A
CP001044	Burkholderia phymatum STM815
AE017333	Bacillus licheniformis DSM 13 = ATCC 14580
AP010655	Streptococcus mutans NN2025
CM000718	Bacillus cereus MM3
CP001034	Natranaerobius thermophilus JW/NM-WN-LF
AE000512	Thermotoga maritima MSB8
CP001022	Exiguobacterium sibiricum 255-15
FM864216	Mycoplasma conjunctivae
CP000563	Shewanella baltica OS155
CP002085	Desulfarculus baarsii DSM 2075
CM000720	Bacillus cereus R309803
CP001940	Desulfurivibrio alkaliphilus AHT2
CP001928	Waddlia chondrophila WSU 86-1044
CP002222	Lactobacillus plantarum subsp. plantarum ST-III
CP001792	Fibrobacter succinogenes subsp. succinogenes S85
CP002170	Mycoplasma hyorhinis HUB-1
CP000409	Rickettsia canadensis str. McKiel
FN434113	Erwinia amylovora CFBP1430
AP009380	Porphyromonas gingivalis ATCC 33277
CP000819	Escherichia coli B str. REL606
CP001707	Kangiella koreensis DSM 16069
FP929140	gamma proteobacterium HdN1
CP001277	Candidatus Hamiltonella defensa 5AT (Acyrthosiphon pisum)
AP012027	Erysipelothrix rhusiopathiae str. Fujisawa
AE017332	Mycoplasma hyopneumoniae 232
AP008971	Finegoldia magna ATCC 29328
CP000437	Francisella tularensis subsp. holarctica OSU18
CP002605	Helicobacter pylori 83
CP000388	Pseudoalteromonas atlantica T6c
CP002657	Alicycliphilus denitrificans K601
CU928160	Escherichia coli IAI1
CP002869	Paenibacillus mucilaginosus KNP414
AP012200	Melissococcus plutonius ATCC 35311
FP929040	Enterobacter cloacae subsp. cloacae NCTC 9394
CP001617	Lactobacillus plantarum JDM1
CP001738	Thermomonospora curvata DSM 43183
CP000859	Desulfococcus oleovorans Hxd3
CP000569	Actinobacillus pleuropneumoniae serovar 5b str. L20
CP001996	Staphylococcus aureus subsp. aureus ED133
FM872307	Chlamydia trachomatis B/TZ1A828/OT
BA000034	Streptococcus pyogenes SSI-1
FM252032	Streptococcus suis BM407
CP002047	Streptomyces bingchenggensis BCW-1
CP002130	Candidatus Midichloria mitochondrii IricVA
FN392235	Erwinia pyrifoliae DSM 12163
CP000025	Campylobacter jejuni RM1221
AP011943	Helicobacter pylori F32
CP001279	Nautilia profundicola AmH
CP002794	Bifidobacterium longum subsp. longum KACC 91563
CP000236	Ehrlichia chaffeensis str. Arkansas
CP001995	Mycoplasma fermentans JER
CM000487	Bacillus subtilis subsp. subtilis str. 168
AP011548	Lactobacillus rhamnosus GG

CP002183	Bacillus subtilis subsp. spizizenii str. W23
AE015451	Pseudomonas putida KT2440
CP002400	Ethanoligenens harbinense YUAN-3
CM000726	Bacillus cereus BDRD-Cer4
AP006840	Symbiobacterium thermophilum IAM 14863
CP000948	Escherichia coli str. K-12 substr. DH10B
AP009493	Streptomyces griseus subsp. griseus NBRC 13350
FQ312030	Streptococcus pneumoniae INV104
CP000393	Trichodesmium erythraeum IMS101
CP000302	Shewanella denitrificans OS217
CP001391	Wolbachia sp. wRi
FP929053	Ruminococcus sp. SR1/5
CP002341	Lactobacillus delbrueckii subsp. bulgaricus ND02
CP002271	Stigmatella aurantiaca DW4/3-1
CP001778	Stackebrandtia nassauensis DSM 44728
CM000789	Mycobacterium tuberculosis KZN R506
CP000264	Jannaschia sp. CCS1
CP001607	Aggregatibacter aphrophilus NJ8700
CP002331	Helicobacter pylori India7
CP002528	Krokinobacter sp. 4H-3-7-5
CM000737	Bacillus cereus AH603
CP001638	Geobacillus sp. WCH70
CP002198	Cyanothece sp. PCC 7822
CP000382	Clostridium novyi NT
CP002006	Prevotella ruminicola 23
CP000301	Rhodopseudomonas palustris BisB18
CP001487	Blattabacterium sp. (Blattella germanica) str. Bge
CP001605	Candidatus Sulcia muelleri SMDSEM
CP000051	Chlamydia trachomatis A/HAR-13
CP002034	Lactobacillus salivarius CECT 5713
CP001364	Chloroflexus sp. Y-400-fl
CP000097	Synechococcus sp. CC9902
BA000018	Staphylococcus aureus subsp. aureus N315
CP000962	Clostridium botulinum A3 str. Loch Maree
AM406671	Lactococcus lactis subsp. cremoris MG1363
CP002458	Mycoplasma fermentans M64
CP002614	Salmonella enterica subsp. enterica serovar Typhimurium str. UK-1
CP000546	Burkholderia mallei NCTC 10229
AE002098	Neisseria meningitidis MC58
CP002552	Nitrosomonas sp. AL212
CP002824	Enterobacter aerogenes KCTC 2190
L42023	Haemophilus influenzae Rd KW20
CP002390	Filifactor alocis ATCC 35896
CP001213	Bifidobacterium animalis subsp. lactis AD011
CP000926	Pseudomonas putida GB-1
CP002028	Thermincola potens JR
CP002330	Caldicellulosiruptor kronotskyensis 2002
CP001853	Bifidobacterium animalis subsp. lactis BB-12
CP001585	Yersinia pestis D106004
FN543502	Citrobacter rodentium ICC168
CP000804	Roseiflexus castenholzii DSM 13941
CP000512	Acidovorax citrulli AAC00-1
CP001791	Bacillus selenitireducens MLS10
CP001672	Methylotenera mobilis JLW8
CP002797	Escherichia coli NA114
CP001252	Shewanella baltica OS223
CP001321	Haemophilus parasuis SH0165
CP001684	Slackia heliotrinireducens DSM 20476
CP000576	Prochlorococcus marinus str. MIT 9301
FP929049	Roseburia intestinalis M50/1
CP001069	Ralstonia pickettii 12J
FP929061	butyrate-producing bacterium SSC/2
FQ312003	Salmonella enterica subsp. enterica serovar Typhimurium str. SL1344
FQ312005	Bacteriovorax marinus SJ
FQ312027	Streptococcus pneumoniae OXC141
AM167904	Bordetella avium 197N
CP000644	Aeromonas salmonicida subsp. salmonicida A449

CP001962	Thermus scotoductus SA-01
CR931997	Corynebacterium jeikeium K411
CP002816	Listeria monocytogenes M7
CP001097	Chlorobium limicola DSM 245
CP001157	Azotobacter vinelandii DJ
CP001033	Streptococcus pneumoniae CGSP14
BA000043	Geobacillus kaustophilus HTA426
CP001020	Coxiella burnetii CbuK˙Q154
FN668944	Clostridium difficile BI9
CP000750	Kineococcus radiotolerans SRS30216
CP001197	Desulfovibrio vulgaris str. 'Miyazaki F'
CP000412	Lactobacillus delbrueckii subsp. bulgaricus ATCC BAA-365
CP000942	Ureaplasma parvum serovar 3 str. ATCC 27815
FP236842	Erwinia pyrifoliae Ep1/96
CP000252	Syntrophus aciditrophicus SB
BA000036	Corynebacterium glutamicum ATCC 13032
CM000735	Bacillus cereus Rock4-18
CP002293	Geobacillus sp. Y4.1MC1
CP000951	Synechococcus sp. PCC 7002
CP002465	Streptococcus suis JS14
CU179680	Mycoplasma agalactiae PG2
CP001900	Campylobacter jejuni subsp. jejuni M1
BX950851	Pectobacterium atrosepticum SCRI1043
CP000524	Bartonella bacilliformis KC583
CP001873	Mycoplasma gallisepticum str. F
CP000449	Maricaulis maris MCS10
FP929052	Ruminococcus champanellensis 18P13
CM000729	Bacillus cereus Rock1-15
CP002154	Edwardsiella tarda FL6-60
CP000930	Heliobacterium modesticaldum Ice1
CP002917	Corynebacterium variabile DSM 44702
CP002924	Corynebacterium pseudotuberculosis PAT10
CP001298	Methylobacterium chloromethanicum CM4
FM200053	Salmonella enterica subsp. enterica serovar Paratyphi A str. AKU˙12601
AP009510	uncultured Termite group 1 bacterium phylotype Rs-D17
AE017308	Mycoplasma mobile 163K
CP002623	Roseobacter litoralis Och 149
BA000021	Wigglesworthia glossinidia endosymbiont of Glossina brevipalpis
CP001604	Listeria monocytogenes 08-5923
FP929062	butyrate-producing bacterium SS3/4
CP000770	Candidatus Sulcia muelleri GWSS
CP000890	Coxiella burnetii RSA 331
CP002025	Brachyspira pilosicoli 95/1000
CP002543	Desulfurobacterium thermolithotrophum DSM 11699
CP001133	Vibrio fischeri MJ11
CP001628	Micrococcus luteus NCTC 2665
CP001631	Acidimicrobium ferrooxidans DSM 10331
CP000653	Enterobacter sp. 638
FP929056	Synergistetes bacterium SGP1
FN665653	Clostridium difficile M120
CP000413	Lactobacillus gasseri ATCC 33323
CR925677	Ehrlichia ruminantium str. Gardel
FR878060	Mycobacterium africanum GM041182
AE017243	Mycoplasma hyopneumoniae J
CP002277	Haemophilus influenzae R2866
CP000488	Candidatus Ruthia magnifica str. Cm (Calyptogena magnifica)
AP008230	Desulfitobacterium hafniense Y51
CP000769	Anaeromyxobacter sp. Fw109-5
CP001146	Dictyoglomus thermophilum H-6-12
CP000721	Clostridium beijerinckii NCIMB 8052
AP009152	Kocuria rhizophila DC2201
CP002224	Ketogulonicigenium vulgare Y25
AE017354	Legionella pneumophila subsp. pneumophila str. Philadelphia 1
CP000931	Shewanella halifaxensis HAW-EB4
BX571966	Burkholderia pseudomallei K96243
CP000473	Candidatus Solibacter usitatus Ellin6076
FR877557	Salmonella bongori NCTC 12419

CP001486	Vibrio cholerae MJ-1236
AP009384	Azorhizobium caulinodans ORS 571
FQ312044	Streptococcus pneumoniae SPN994039
CP001220	Comamonas testosteroni CNB-2
CP001905	Thioalkalivibrio sp. K90mix
CP002804	Chlamydophila psittaci C19/98
FQ790233	Mycoplasma suis KI3806
CP002280	Rothia dentocariosa ATCC 17931
CP002097	Corynebacterium pseudotuberculosis FRC41
CP000703	Staphylococcus aureus subsp. aureus JH9
CP002478	Staphylococcus pseudintermedius ED99
CP002743	Bifidobacterium breve ACS-071-V-Sch8b
CP002077	Mycoplasma pneumoniae FH
CP002346	Riemerella anatipestifer ATCC 11845 = DSM 15868
CP000285	Chromohalobacter salexigens DSM 3043
CU466930	Candidatus Cloacamonas acidaminovorans str. Evry
CP000016	Candidatus Blochmannia pennsylvanicus str. BPEN
CU458896	Mycobacterium abscessus
CP000717	Mycobacterium tuberculosis F11
CP002054	Chlamydia trachomatis D-LC
CP002549	Chlamydophila psittaci 6BC
CP001739	Sebaldella termitidis ATCC 33386
CP001612	Rickettsia africae ESF-5
CP000239	Synechococcus sp. JA-3-3Ab
CP002421	Neisseria meningitidis M01-240149
AE009440	Chlamydophila pneumoniae TW-183
CP001666	Clostridium ljungdahlii DSM 13528
CP001013	Leptothrix cholodnii SP-6
CP001275	Thermomicrobium roseum DSM 5159
CP000487	Campylobacter fetus subsp. fetus 82-40
CP000144	Rhodobacter sphaeroides 2.4.1
CP000949	Pseudomonas putida W619
CP001746	Bacillus cereus biovar anthracis str. CI
CP000555	Methylibium petroleiphilum PM1
AL111168	Campylobacter jejuni subsp. jejuni NCTC 11168 = ATCC 700819
CP002011	Clostridium botulinum F str. 230613
CP001175	Listeria monocytogenes HCC23
FN666575	Erwinia amylovora ATCC 49946
CP000158	Hyphomonas neptunium ATCC 15444
CP001619	Dyadobacter fermentans DSM 18053
CP002104	Gardnerella vaginalis ATCC 14019
CP000730	Staphylococcus aureus subsp. aureus USA300˙TCH1516
CP001677	Candidatus Liberibacter asiaticus str. psy62
CP001215	Bacillus anthracis str. CDC 684
CP000245	Ramlibacter tataouinensis TTB310
FP475956	Thiomonas sp. 3As
CP000482	Pelobacter propionicus DSM 2379
CP001185	Thermosipho africanus TCF52B
CP000671	Haemophilus influenzae PittEE
CP000849	Rickettsia bellii OSU 85-389
FR873482	Streptococcus salivarius JIM8777
AP011156	Acetobacter pasteurianus IFO 3283-32
CM000439	Burkholderia thailandensis E264
CP000024	Streptococcus thermophilus CNRZ1066
CP000394	Granulibacter bethesdensis CGDNIH1
CP002216	Caldicellulosiruptor owensensis OL
CP002522	Acinetobacter baumannii TCDC-AB0715
FP103042	Methylobacterium extorquens DM4
CP002630	Marinithermus hydrothermalis DSM 14884
CP002466	Thermoanaerobacter brockii subsp. finnii Ako-1
CM000770	Rickettsia endosymbiont of Ixodes scapularis
AP009387	Burkholderia multivorans ATCC 17616
AP006841	Bacteroides fragilis YCH46
CP001819	Sanguibacter keddieii DSM 10542
CP000438	Pseudomonas aeruginosa UCBPP-PA14
CP002773	Serratia plymuthica AS9
CP001144	Salmonella enterica subsp. enterica serovar Dublin str. CT˙02021853

DQ489736	Leuconostoc citreum KM20
CP002113	Capnocytophaga canimorsus Cc5
CP002033	Lactobacillus fermentum CECT 5716
AE013218	Buchnera aphidicola str. Sg (Schizaphis graminum)
CP000312	Clostridium perfringens SM101
CM000743	Bacillus mycoides Rock1-4
FP929039	Coprococcus sp. ART55/1
CP001983	Bacillus megaterium QM B1551
AE004969	Neisseria gonorrhoeae FA 1090
CP000233	Lactobacillus salivarius UCC118
CP002455	Weeksella virosa DSM 16922
AE016853	Pseudomonas syringae pv. tomato str. DC3000
AE017042	Yersinia pestis biovar Microtus str. 91001
CP002459	Brucella melitensis M28
CP000448	Syntrophomonas wolfei subsp. wolfei str. Goettingen
CP002764	Lactobacillus kefiranofaciens ZW3
CP002326	Caldicellulosiruptor kristjanssonii 177R1B
CP002279	Mesorhizobium opportunistum WSM2075
CP002371	Candidatus Liberibacter solanacearum CLso-ZC1
AE017143	Haemophilus ducreyi 35000HP
CP000612	Desulfotomaculum reducens MI-1
CP000012	Helicobacter pylori 51
CP001921	Acinetobacter baumannii 1656-2
CP001854	Conexibacter woesei DSM 14684
CP000884	Delftia acidovorans SPH-1
CP002052	Chlamydia trachomatis D-EC
CP000381	Neisseria meningitidis 053442
CP002083	Hyphomicrobium denitrificans ATCC 51888
CP002329	Mycobacterium sp. JDM601
BX470251	Photorhabdus luminescens subsp. laumondii TTO1
AM494475	Orientia tsutsugamushi str. Boryong
AE003852	Vibrio cholerae O1 biovar El Tor str. N16961
CP001589	Yersinia pestis D182038
CP000759	Ochrobactrum anthropi ATCC 49188
CP001968	Denitrovibrio acetiphilus DSM 12809
CM000741	Bacillus cereus AH1273
CP000001	Bacillus cereus E33L
CP000383	Cytophaga hutchinsonii ATCC 33406
CP000441	Burkholderia ambifaria AMMD
BX548020	Synechococcus sp. WH 8102
CR954247	Pseudoalteromonas haloplanktis TAC125
CP000107	Ehrlichia canis str. Jake
CP001132	Acidithiobacillus ferrooxidans ATCC 53993
BA000026	Mycoplasma penetrans HF-2
CP001063	Shigella boydii CDC 3083-94
BX936398	Yersinia pseudotuberculosis IP 32953
AP010904	Desulfovibrio magneticus RS-1
CP002643	Staphylococcus aureus subsp. aureus T0131
CM000750	Bacillus thuringiensis serovar pakistani str. T13001
CP001339	Thioalkalivibrio sulfidophilus HL-EbGr7
CP001091	Actinobacillus pleuropneumoniae serovar 7 str. AP76
CP000786	Leptospira biflexa serovar Patoc strain 'Patoc 1 (Paris)'
CM000732	Bacillus cereus Rock3-42
AE017282	Methylococcus capsulatus str. Bath
CP000661	Rhodobacter sphaeroides ATCC 17025
CP001561	Neisseria meningitidis alpha710
CP001621	Mycoplasma mycoides subsp. capri str. GM12
CP002286	Bifidobacterium longum subsp. longum BBMN68
CP000716	Thermosipho melanesiensis BI429
CP000414	Leuconostoc mesenteroides subsp. mesenteroides ATCC 8293
CM000753	Bacillus thuringiensis serovar berliner ATCC 10792
AM260479	Ralstonia eutropha H16
FP929050	Roseburia intestinalis XB6B4
CP001129	Streptococcus equi subsp. zooepidemicus MGCS10565
CP002039	Herbaspirillum seropedicae SmR1
CP001678	Hirschia baltica ATCC 49814
CP001720	Desulfotomaculum acetoxidans DSM 771

CU914168	Ralstonia solanacearum IPO1609
CP001080	Sulfurihydrogenibium sp. YO3AOP1
CP002618	Lactobacillus casei BD-II
FR873481	Streptococcus salivarius CCHSS3
CP002399	Micromonospora sp. L5
CP001052	Burkholderia phytofirmans PsJN
CP001811	Butyrivibrio proteoclasticus B316
AP010889	Bifidobacterium longum subsp. infantis ATCC 15697 = JCM 1222
CP000544	Halorhodospira halophila SL1
CP001078	Clostridium botulinum E3 str. Alaska E43
CP002050	Geobacillus sp. C56-T3
AE017223	Brucella abortus bv. 1 str. 9-941
CP001511	Methylobacterium extorquens AM1
CP001632	Capnocytophaga ochracea DSM 7271
CP000611	Mycobacterium tuberculosis H37Ra
FM177140	Lactobacillus casei BL23
AE017197	Rickettsia typhi str. Wilmington
CP000923	Thermoanaerobacter sp. X514
CP002165	Xylella fastidiosa subsp. fastidiosa GB514
AM260522	Helicobacter acinonychis str. Sheeba
CP000230	Rhodospirillum rubrum ATCC 11170
FM999788	Neisseria meningitidis 8013
BX470248	Bordetella pertussis Tohama I
CP000444	Shewanella sp. MR-7
CP000492	Chlorobium phaeobacteroides DSM 266
AE004091	Pseudomonas aeruginosa PAO1
CP001685	Leptotrichia buccalis C-1013-b
CP001472	Acidobacterium capsulatum ATCC 51196
CP000490	Paracoccus denitrificans PD1222
CP000857	Salmonella enterica subsp. enterica serovar Paratyphi C strain RKS4594
CP001965	Sideroxydans lithotrophicus ES-1
CP000672	Haemophilus influenzae PittGG
CP000156	Lactobacillus delbrueckii subsp. bulgaricus 2038
CP001807	Rhodothermus marinus DSM 4252
CU207366	Gramella forsetii KT0803
CP000259	Streptococcus pyogenes MGAS9429
CP000410	Streptococcus pneumoniae D39
CP001084	Lactobacillus casei str. Zhang
FN563149	Rhodococcus equi 103S
CP001649	Desulfovibrio salexigens DSM 2638
CP001671	Escherichia coli ABU 83972
CP001907	Bacillus thuringiensis serovar chinensis CT-43
CP002870	Pseudomonas putida S16
CP000915	Francisella tularensis subsp. mediasiatica FSC147
CP001829	Corynebacterium pseudotuberculosis C231
AE014299	Shewanella oneidensis MR-1
AE016827	Mannheimia succiniciproducens MBEL55E
CP002637	Selenomonas sputigena ATCC 35185
CP000325	Mycobacterium ulcerans Agy99
AP007255	Magnetospirillum magneticum AMB-1
CP001408	Burkholderia pseudomallei MSHR346
CP002620	Pseudomonas mendocina NK-01
AM711867	Clavibacter michiganensis subsp. michiganensis NCPPB 382
CP000385	Mycobacterium sp. MCS
CP002156	Parvularcula bermudensis HTCC2503
CM000441	Clostridium difficile QCD-66c26
CP000683	Rickettsia massiliae MTU5
CP001085	Candidatus Riesia pediculicola USDA
BX470250	Bordetella bronchiseptica RB50
CP002355	Sulfuricurvum kujiense DSM 16994
CP002899	Weissella koreensis KACC 15510
CP000157	Erythrobacter litoralis HTCC2594
CP002691	Haliscomenobacter hydrossis DSM 1100
FP929046	Faecalibacterium prausnitzii SL3/3
CP001999	Arcobacter nitrofigilis DSM 7299
CP000746	Actinobacillus succinogenes 130Z
CP001681	Pedobacter heparinus DSM 2366

CP000767	Campylobacter curvus 525.92
CM000717	Bacillus cereus 172560W
CP001924	Dehalococcoides sp. GT
CP001698	Spirochaeta thermophila DSM 6192
CP000152	Burkholderia sp. 383
CP002272	Enterobacter cloacae SCF1
CP001751	Candidatus Puniceispirillum marinum IMCC1322
CP001966	Tsukamurella paurometabola DSM 20162
CP000656	Mycobacterium gilvum PYR-GCK
CP001806	Vibrio sp. Ex25
CP000038	Shigella sonnei Ss046
FR824043	Streptococcus gallolyticus subsp. gallolyticus ATCC BAA-2069
AM408590	Mycobacterium bovis BCG str. Pasteur 1173P2
CP002589	Prevotella denticola F0289
CP000941	Xylella fastidiosa M12
CP000912	Brucella suis ATCC 23445
CP002844	Lactobacillus reuteri SD2112
CP002480	Granulicella tundricola MP5ACTX9
CP001834	Lactococcus lactis subsp. lactis KF147
CP000323	Psychrobacter cryohalolentis K5
AE017262	Listeria monocytogenes serotype 4b str. F2365
FP929054	Ruminococcus obeum A2-162
CP002163	Candidatus Sulcia muelleri CARI
FQ670179	Helicobacter felis ATCC 49179
CP000308	Yersinia pestis Antiqua
CM000488	Bacillus subtilis subsp. subtilis str. NCIB 3610
CP000511	Mycobacterium vanbaalenii PYR-1
CP002114	Staphylococcus aureus subsp. aureus JKD6159
CP000830	Dinoroseobacter shibae DFL 12
AP006627	Bacillus clausii KSM-K16
CP002157	Maribacter sp. HTCC2170
FR871757	Helicobacter bizzozeronii CIII-1
CP000554	Prochlorococcus marinus str. MIT 9303
CP002365	Lactococcus lactis subsp. lactis CV56
CM000730	Bacillus cereus Rock3-28
FM209186	Pseudomonas aeruginosa LESB58
CM000751	Bacillus thuringiensis serovar kurstaki str. T03a001
CU459141	Acinetobacter baumannii AYE
BX908798	Candidatus Protochlamydia amoebophila UWE25
FM204883	Streptococcus equi subsp. equi 4047
FM211688	Listeria monocytogenes L99
CP001010	Polynucleobacter necessarius subsp. necessarius STIR1
CP002815	Propionibacterium acnes 6609
FN667742	Xenorhabdus nematophila ATCC 19061
BA000022	Synechocystis sp. PCC 6803
CP000605	Bifidobacterium longum DJO10A
CM000788	Mycobacterium tuberculosis KZN V2475
CP001344	Cyanothece sp. PCC 7425
CP002106	Olsenella uli DSM 7084
CP001682	Cryptobacterium curtum DSM 15641
CP000116	Thiobacillus denitrificans ATCC 25259
FR870271	Staphylococcus lugdunensis N920143
FP476056	Zobellia galactanivorans
CP002161	Candidatus Zinderia insecticola CARI
CP001662	Mycobacterium tuberculosis KZN 4207
CP000724	Alkaliphilus metalliredigens QYMF
CR543861	Acinetobacter sp. ADP1
CP000009	Gluconobacter oxydans 621H
CP000790	Vibrio harveyi ATCC BAA-1116
CP000829	Streptococcus pyogenes NZ131
CM000662	Escherichia coli O157
FR856861	Novosphingobium sp. PP1Y
CP002429	Lactobacillus helveticus H10
CP001804	Haliangium ochraceum DSM 14365
CP000263	Buchnera aphidicola BCc
CP002918	Candidatus Tremblaya princeps PCVAL
FQ312002	Haemophilus parainfluenzae T3T1

FP929041	Eubacterium cylindroides T2-87
CP001510	Methylobacterium extorquens AM1
CP002073	Helicobacter pylori SJM180
CP002776	Thioalkalimicrobium cyclicum ALM1
CP001095	Bifidobacterium longum subsp. infantis ATCC 15697 = JCM 1222
CP000513	Dichelobacter nodosus VCS1703A
CP002206	Pantoea vagans C9-1
CP002042	Meiothermus silvanus DSM 9946
CP001606	Bifidobacterium animalis subsp. lactis DSM 10140
AP010890	Bifidobacterium longum subsp. infantis 157F
AM999887	Wolbachia endosymbiont of Culex quinquefasciatus Pel
BA000031	Vibrio parahaemolyticus RIMD 2210633
CP002046	Croceibacter atlanticus HTCC2559
AF222894	Ureaplasma parvum serovar 3 str. ATCC 700970
CP000921	Streptococcus pneumoniae Taiwan19F-14
CP001641	Mycobacterium tuberculosis CCDC5079
CP000518	Mycobacterium sp. KMS
FN538970	Clostridium difficile CD196
AM286690	Alcanivorax borkumensis SK2
AP009049	Clostridium kluyveri NBRC 12016
CM000745	Bacillus pseudomycoides DSM 12442
CP001176	Bacillus cereus B4264
CP000468	Escherichia coli APEC O1
CP002791	Corynebacterium ulcerans BR-AD22
CP002736	Desulfotomaculum carboxydivorans CO-1-SRB
CP000446	Shewanella sp. MR-4
CP002045	Arcanobacterium haemolyticum DSM 20595
FQ312039	Streptococcus pneumoniae SPN032672
CP000113	Myxococcus xanthus DK 1622
FQ312043	Streptococcus pneumoniae SPN034183
AM181176	Pseudomonas fluorescens SBW25
CP000851	Shewanella pealeana ATCC 700345
CP001015	Streptococcus pneumoniae G54
CP001615	Exiguobacterium sp. AT1b
CP000822	Citrobacter koseri ATCC BAA-895
CP002292	Rhodomicrobium vannielii ATCC 17100
CP001029	Methylobacterium populi BJ001
CP000112	Desulfovibrio alaskensis G20
BX927147	Corynebacterium glutamicum ATCC 13032
BX248583	Candidatus Blochmannia floridanus
CP000807	Cyanothece sp. ATCC 51142
AP006716	Staphylococcus haemolyticus JCSC1435
AM180355	Clostridium difficile 630
BA000030	Streptomyces avermitilis MA-4680
CP001147	Thermodesulfovibrio yellowstonii DSM 11347
CP002609	Lactobacillus amylovorus GRL1118
CP002423	Neisseria meningitidis M04-240196
AM946981	Escherichia coli BL21(DE3)
AE017225	Bacillus anthracis str. Sterne
CP001280	Methylocella silvestris BL2
CP000423	Lactobacillus casei ATCC 334
CP002546	Planctomyces brasiliensis DSM 5305
AP008229	Xanthomonas oryzae pv. oryzae MAFF 311018
CP001781	Staphylococcus aureus subsp. aureus ED98
CP000271	Burkholderia xenovorans LB400
FR720602	Streptococcus oralis Uo5
CM000657	Clostridium difficile QCD-97b34
CP002074	Helicobacter pylori PeCan4
FN668941	Clostridium difficile BI1
CP001396	Escherichia coli BW2952
CP000269	Janthinobacterium sp. Marseille
CP000089	Dechloromonas aromatica RCB
CP000303	Bifidobacterium breve UCC2003
CP001349	Methylobacterium nodulans ORS 2060
AE015925	Chlamydophila caviae GPIC
CP002209	Ferrimonas balearica DSM 9799
CR628336	Legionella pneumophila str. Paris

CP002005	Moraxella catarrhalis BBH18
CP002770	Desulfotomaculum kuznetsovii DSM 6115
CP001875	Pantoea ananatis LMG 20103
CP002332	Helicobacter pylori Gambia94/24
CP002049	Truepera radiovictrix DSM 17093
CP000509	Nocardioides sp. JS614
CP002826	Oligotropha carboxidovorans OM5
CP002573	Acidithiobacillus caldus SM-1
CP000353	Cupriavidus metallidurans CH34
FM179322	Lactobacillus rhamnosus GG
CP000812	Thermotoga lettingae TMO
CP000462	Aeromonas hydrophila subsp. hydrophila ATCC 7966
CP001629	Desulfomicrobium baculatum DSM 4028
CM000740	Bacillus cereus AH1272
BA000016	Clostridium perfringens str. 13
AM412317	Clostridium botulinum A str. ATCC 3502
CP002586	Chlamydophila psittaci 6BC
CP002872	Francisella sp. TX077308
CP002276	Haemophilus influenzae R2846
CP001733	Aggregatibacter actinomycetemcomitans D11S-1
CP000939	Clostridium botulinum B1 str. Okra
AP011170	Acetobacter pasteurianus IFO 3283-12
CT573213	Frankia alni ACN14a
CM000659	Clostridium difficile CIP 107932
CP001079	Anaplasma marginale str. Florida
CP000733	Coxiella burnetii Dugway 5J108-111
CP002301	Buchnera aphidicola str. TLW03 (Acyrthosiphon pisum)
AP010960	Escherichia coli O111
CM000721	Bacillus cereus ATCC 4342
CP001642	Mycobacterium tuberculosis CCDC5180
CP002305	Leadbetterella byssophila DSM 17132
CP002394	Bacillus cellulosilyticus DSM 2522
CP001120	Salmonella enterica subsp. enterica serovar Heidelberg str. SL476
AE009951	Fusobacterium nucleatum subsp. nucleatum ATCC 25586
CP001135	Edwardsiella tarda EIB202
AP010946	Azospirillum sp. B510
CP001637	Escherichia coli DH1
CP001936	Thermoanaerobacter italicus Ab9
CP000013	Borrelia garinii PBi
AP009048	Escherichia coli str. K-12 substr. W3110
CP002131	Thermosediminibacter oceani DSM 16646
CP002032	Thermoanaerobacter mathranii subsp. mathranii str. A3
AE017285	Desulfovibrio vulgaris str. Hildenborough
CP001189	Gluconacetobacter diazotrophicus PAl 5
CP000390	Chelativorans sp. BNC1
CP001785	Ammonifex degensii KC4
AP009179	Sulfurovum sp. NBC37-1
CM000855	Campylobacter jejuni subsp. jejuni 414
CP002076	Helicobacter pylori Cuz20
CP001726	Eggerthella lenta DSM 2243
FN554889	Streptomyces scabiei 87.22
AE004092	Streptococcus pyogenes M1 GAS
CP000976	Borrelia duttonii Ly
CP000736	Staphylococcus aureus subsp. aureus JH1
AM946016	Streptococcus suis P1/7
CP002606	Hippea maritima DSM 10411
CM000787	Mycobacterium tuberculosis KZN 4207
CP000738	Sinorhizobium medicae WSM419
CP000439	Francisella novicida U112
AE000657	Aquifex aeolicus VF5
CP001099	Chlorobaculum parvum NCIB 8327
BX842601	Bdellovibrio bacteriovorus HD100
CP002290	Pseudomonas putida BIRD-1
CP000123	Mycoplasma capricolum subsp. capricolum ATCC 27343
BA000003	Buchnera aphidicola str. APS (Acyrthosiphon pisum)
CP001600	Edwardsiella ictaluri 93-146
AP009153	Gemmatimonas aurantiaca T-27

CP002340	Streptococcus thermophilus ND03
CP001816	Sulfurospirillum deleyianum DSM 6946
CP002123	Prevotella melaninogenica ATCC 25845
AL645882	Streptomyces coelicolor A3(2)
AE001273	Chlamydia trachomatis D/UW-3/CX
CP001998	Coraliomargarita akajimensis DSM 45221
CP001701	Cyanothece sp. PCC 8802
AM263198	Listeria welshimeri serovar 6b str. SLCC5334
CP000380	Burkholderia cenocepacia AU 1054
AL591688	Sinorhizobium meliloti 1021
CP000771	Fervidobacterium nodosum Rt17-B1
FM211192	Mycobacterium leprae Br4923
CP002349	Marivirga tractuosa DSM 4126
CP002252	Methylovorus sp. MP688
CP002471	Streptococcus parauberis KCTC 11537
AJ965256	Dehalococcoides sp. CBDB1
CP000527	Desulfovibrio vulgaris DP4
CP002607	Aeromonas veronii B565
CP001164	Escherichia coli O157
FQ312041	Streptococcus pneumoniae SPN994038
CP001089	Geobacter lovleyi SZ
CP002221	Hydrogenobacter thermophilus TK-6
CR936503	Lactobacillus sakei subsp. sakei 23K
CP002243	Candidatus Moranella endobia PCIT
CP000766	Rickettsia rickettsii str. Iowa
CM000440	Fusobacterium nucleatum subsp. polymorphum ATCC 10953
CP002176	Streptococcus pneumoniae 670-6B
CP000260	Streptococcus pyogenes MGAS10270
CP000086	Burkholderia thailandensis E264
FP565809	[Clostridium] sticklandii
AM295007	Streptococcus pyogenes str. Manfredo
CP001283	Bacillus cereus AH820
CP000878	Prochlorococcus marinus str. MIT 9211
CP000879	Petrotoga mobilis SJ95
CP000284	Methylobacillus flagellatus KT
CP002511	Candidatus Pelagibacter sp. IMCC9063
CP000083	Colwellia psychrerythraea 34H
CP000925	synthetic Mycoplasma genitalium JCVI-1.0
CP002821	Oligotropha carboxidovorans OM4
AE017220	Salmonella enterica subsp. enterica serovar Choleraesuis str. SC-B67
CP001172	Acinetobacter baumannii AB307-0294
CP002353	Isosphaera pallida ATCC 43644
FM178380	Aliivibrio salmonicida LFI1238
CP001340	Caulobacter crescentus NA1000
CP000463	Rhodopseudomonas palustris BisA53
AP011112	Hydrogenobacter thermophilus TK-6
AL732656	Streptococcus agalactiae NEM316
CP000959	Burkholderia cenocepacia MC0-3
AE017125	Helicobacter hepaticus ATCC 51449
CP001072	Helicobacter pylori Shi470
CP000480	Mycobacterium smegmatis str. MC2 155
CP001992	Mobiluncus curtisii ATCC 43063
CP000232	Moorella thermoacetica ATCC 39073
CM000637	Clostridium difficile QCD-63q42
CP002888	Streptococcus salivarius 57.I
CP001991	Mycoplasma crocodyli MP145
CP002385	Mycobacterium gilvum Spyr1
CP002116	Spirochaeta smaragdinae DSM 11293
CP001655	Dickeya zeae Ech1591
CT971583	Synechococcus sp. WH 7803
CP000503	Shewanella sp. W3-18-1
CU928163	Escherichia coli UMN026
AP011940	Helicobacter pylori F16
CM000287	Clostridium difficile QCD-32g58
CP000880	Salmonella enterica subsp. arizonae serovar 62
CP000848	Rickettsia rickettsii str. 'Sheila Smith'
AM180252	Lawsonia intracellularis PHE/MN1-00

CP000521	Acinetobacter baumannii ATCC 17978
CP002297	Desulfovibrio vulgaris RCH1
CR767821	Ehrlichia ruminantium str. Welgevonden
FN298497	Lactobacillus johnsonii FI9785
FM954973	Vibrio splendidus LGP32
CP002545	Pedobacter saltans DSM 12145
BA000017	Staphylococcus aureus subsp. aureus Mu50
CP001368	Escherichia coli O157
AP009180	Candidatus Carsonella ruddii PV
FP929060	butyrate-producing bacterium SM4/1
FP929058	Enterococcus sp. 7L76
FP565575	Candidatus Methylomirabilis oxyfera
CP001840	Bifidobacterium bifidum PRL2010
AE008923	Xanthomonas axonopodis pv. citri str. 306
AM747721	Burkholderia cenocepacia J2315
CP001110	Pelodictyon phaeoclathratiforme BU-1
BA000028	Oceanobacillus iheyensis HTE831
AP011121	Acetobacter pasteurianus IFO 3283-01
CP000243	Escherichia coli UTI89
CP000781	Xanthobacter autotrophicus Py2
CP001096	Rhodopseudomonas palustris TIE-1
CP002210	Thermoanaerobacter sp. X513
FN557490	Listeria seeligeri serovar 1/2b str. SLCC3954
CP000817	Lysinibacillus sphaericus C3-41
BX571856	Staphylococcus aureus subsp. aureus MRSA252
FM162591	Photorhabdus asymbiotica
CP000726	Clostridium botulinum A str. ATCC 19397
CP000825	Prochlorococcus marinus str. MIT 9215
CP000356	Sphingopyxis alaskensis RB2256
CP002774	Serratia sp. AS12
CP002102	Brevundimonas subvibrioides ATCC 15264
CP002665	[Cellvibrio] gilvus ATCC 13127
CP000082	Psychrobacter arcticus 273-4
CP000747	Phenylobacterium zucineum HLK1
CP001790	Pectobacterium wasabiae WPP163
CP000050	Xanthomonas campestris pv. campestris str. 8004
CP000474	Arthrobacter aurescens TC1
CP002547	Syntrophobotulus glycolicus DSM 8271
CP002010	Bifidobacterium longum subsp. longum JDM301
CP002071	Helicobacter pylori Sat464
CM000746	Bacillus thuringiensis serovar tochigiensis BGSC 4Y1
CP000319	Nitrobacter hamburgensis X14
CP002512	Aerococcus urinae ACS-120-V-Col10a
CP001127	Salmonella enterica subsp. enterica serovar Schwarzengrund str. CVM19633
CP000993	Borrelia recurrentis A1
FP929048	Megamonas hypermegale ART12/1
CP000481	Acidothermus cellulolyticus 11B
FQ482149	Chlamydophila psittaci RD1
AE009948	Streptococcus agalactiae 2603V/R
AE014184	Tropheryma whipplei str. Twist
CP001644	Ralstonia pickettii 12D
FP929045	Faecalibacterium prausnitzii L2-6
CP000571	Burkholderia pseudomallei 668
CP000507	Shewanella amazonensis SB2B
L43967	Mycoplasma genitalium G37
CP001658	Mycobacterium tuberculosis KZN 1435
CP000776	Campylobacter hominis ATCC BAA-381
CM000833	Burkholderia pseudomallei 1710a
AP008934	Staphylococcus saprophyticus subsp. saprophyticus ATCC 15305
CP002084	Dehalogenimonas lykanthroporepellens BL-DC-9
CP002467	Terriglobus saanensis SP1PR4
FN554766	Escherichia coli 042
AL445566	Mycoplasma pulmonis UAB CTIP
CP000250	Rhodopseudomonas palustris HaA2
CP002454	Deinococcus maricopensis DSM 21211
CP001721	Atopobium parvulum DSM 20469
CP002457	Shewanella putrefaciens 200

CP001665	Escherichia coli 'BL21-Gold(DE3)pLysS AG'
CP000450	Nitrosomonas eutropha C91
CP002000	Amycolatopsis mediterranei U32
CP000712	Pseudomonas putida F1
CP000753	Shewanella baltica OS185
FN597254	Streptococcus gallolyticus UCN34
CP001602	Listeria monocytogenes 08-5578
CP002850	Zymomonas mobilis subsp. mobilis ATCC 10988
CM000752	Bacillus thuringiensis serovar monterrey BGSC 4AJ1
CP000238	Baumannia cicadellinicola str. Hc (Homalodisca coagulata)
CP002446	Pseudoxanthomonas suwonensis 11-1
CP000526	Burkholderia mallei SAVP1
CP002652	Lactobacillus buchneri NRRL B-30929
CP001431	Neorickettsia risticii str. Illinois
CP001359	Anaeromyxobacter dehalogenans 2CP-1
AE015928	Bacteroides thetaiotaomicron VPI-5482
CP002902	Alicyclobacillus acidocaldarius subsp. acidocaldarius Tc-4-1
BX248333	Mycobacterium bovis AF2122/97
FN692037	Lactobacillus crispatus ST1
AP009351	Staphylococcus aureus subsp. aureus str. Newman
CT978603	Synechococcus sp. RCC307
FP885891	Ralstonia solanacearum PSI07
CP001229	Sulfurihydrogenibium azorense Az-Fu1
AE003849	Xylella fastidiosa 9a5c
CP000687	Actinobacillus pleuropneumoniae serovar 3 str. JL03
AL450380	Mycobacterium leprae TN
BA000037	Vibrio vulnificus YJ016
CP000728	Clostridium botulinum F str. Langeland
CP002433	Pantoea sp. At-9b
CP000964	Klebsiella pneumoniae 342
CP001103	Alteromonas macleodii str. 'Deep ecotype'
CP000922	Anoxybacillus flavithermus WK1
AE009949	Streptococcus pyogenes MGAS8232
AM743169	Stenotrophomonas maltophilia K279a
CP000479	Mycobacterium avium 104
CP002508	Bacillus thuringiensis serovar finitimus YBT-020
CP000469	Shewanella sp. ANA-3
AM039952	Xanthomonas campestris pv. vesicatoria str. 85-10
CP002727	Pseudomonas fulva 12-X
CP002038	Dickeya dadantii 3937
AP011163	Acetobacter pasteurianus IFO 3283-01-42C
CP001827	Dehalococcoides sp. VS
CP000435	Synechococcus sp. CC9311
CP000920	Streptococcus pneumoniae P1031
CP001584	Rickettsia prowazekii Rp22
CP000305	Yersinia pestis Nepal516
CP000860	Candidatus Desulforudis audaxviator MP104C
CP001593	Yersinia pestis Z176003
CP002444	Thermovibrio ammonificans HB-1
CP001055	Elusimicrobium minutum Pei191
CP000478	Syntrophobacter fumaroxidans MPOB
AP011128	Acetobacter pasteurianus IFO 3283-03
CP001226	Candidatus Hodgkinia cicadicola Dsem
CP000891	Shewanella baltica OS195
AP012052	Microbacterium testaceum StLB037
CP000240	Synechococcus sp. JA-2-3B'a(2-13)
CU207211	Herminiimonas arsenicoxydans
AE009952	Yersinia pestis KIM10+
AP011177	Shewanella violacea DSS12
CP001227	Rickettsia peacockii str. Rustic
CP000805	Treponema pallidum subsp. pallidum SS14
CP002352	Bacteroides helcogenes P 36-108
CP001700	Catenulispora acidiphila DSM 44928
CP001874	Thermobispora bispora DSM 43833
CM000660	Clostridium difficile QCD-23m63
CP000431	Rhodococcus jostii RHA1
CR925678	Ehrlichia ruminantium str. Welgevonden

CP001102	Candidatus Amoebophilus asiaticus 5a2
CR954253	Lactobacillus delbrueckii subsp. bulgaricus ATCC 11842
CP001708	Anaerococcus prevotii DSM 20548
CM000489	Bacillus subtilis subsp. subtilis str. JH642
AM946015	Streptococcus uberis 0140J
CP002017	Kyrpidia tusciae DSM 2912
CP000453	Alkalilimnicola ehrlichii MLHE-1
CP000557	Geobacillus thermodenitrificans NG80-2
CP001363	Salmonella enterica subsp. enterica serovar Typhimurium str. 14028S
CP001219	Acidithiobacillus ferrooxidans ATCC 23270
CP002557	Francisella cf. novicida Fx1
CP002629	Desulfobacca acetoxidans DSM 11109
CM000725	Bacillus cereus BDRD-ST196
CP000934	Cellvibrio japonicus Ueda107
CP002303	Buchnera aphidicola str. JF98 (Acyrthosiphon pisum)
FP929003	Candidatus Nitrospira defluvii
FR845719	Streptomyces venezuelae ATCC 10712
CP002904	Streptococcus equi subsp. zooepidemicus ATCC 35246
AE016879	Bacillus anthracis str. Ames
BX571656	Wolinella succinogenes DSM 1740
CP002775	Serratia sp. AS13
AP012212	Clostridium sp. SY8519
AE007870	Agrobacterium fabrum str. C58
CP000084	Candidatus Pelagibacter ubique HTCC1062
CP001887	Chlamydia trachomatis G/9768
AP011115	Rhodococcus opacus B4
CP000607	Chlorobium phaeovibrioides DSM 265
CM000749	Bacillus thuringiensis serovar sotto str. T04001
CP001743	Meiothermus ruber DSM 1279
BA000007	Escherichia coli O157
CP000485	Bacillus thuringiensis str. Al Hakam
CP000235	Anaplasma phagocytophilum HZ
CP001108	Prosthecochloris aestuarii DSM 271
CP000886	Salmonella enterica subsp. enterica serovar Paratyphi B str. SPB7
CP000261	Streptococcus pyogenes MGAS2096
CP000010	Burkholderia mallei ATCC 23344
CM000722	Bacillus cereus m1550
CP002583	Marinomonas mediterranea MMB-1
CP000961	Shewanella woodyi ATCC 51908
CP000034	Shigella dysenteriae Sd197
CP002452	Nitratifractor salsuginis DSM 16511
CP002808	Mycoplasma haemofelis Ohio2
FR774048	Neisseria meningitidis WUE 2594
CP001849	Gardnerella vaginalis 409-05
CP002859	Runella slithyformis DSM 19594
CP002021	Thiomonas intermedia K12
AE017321	Wolbachia endosymbiont strain TRS of Brugia malayi
CP000936	Streptococcus pneumoniae Hungary19A-6
CP001654	Dickeya dadantii Ech703
BA000039	Thermosynechococcus elongatus BP-1
CP000764	Bacillus cytotoxicus NVH 391-98
CP000142	Pelobacter carbinolicus DSM 2380
CP002696	Treponema brennaborense DSM 12168
CM000719	Bacillus cereus AH621
CP001341	Arthrobacter chlorophenolicus A6
AJ938182	Staphylococcus aureus RF122
CP001683	Saccharomonospora viridis DSM 43017
CP000304	Pseudomonas stutzeri A1501
CP001795	Geobacillus sp. Y412MC61
CP001622	Rhizobium leguminosarum bv. trifolii WSM1325
CP000916	Thermotoga neapolitana DSM 4359
CU914166	Ralstonia solanacearum IPO1609
CP000033	Lactobacillus acidophilus NCFM
CP000668	Yersinia pestis Pestoides F
CP000580	Mycobacterium sp. JLS
CP002896	Amycolatopsis mediterranei S699
CP000655	Polynucleobacter necessarius subsp. asymbioticus QLW-P1DMWA-1

AM260525	Bartonella tribocorum CIP 105476
CP002738	Methylomonas methanica MC09
CP002735	Delftia sp. Cs1-4
CP001087	Desulfobacterium autotrophicum HRM2
AE016830	Enterococcus faecalis V583
CP001186	Bacillus cereus G9842
CP000027	Dehalococcoides ethenogenes 195
CP000713	Psychrobacter sp. PRwf-1
CP002347	Calditerrivibrio nitroreducens DSM 19672
AE014613	Salmonella enterica subsp. enterica serovar Typhi str. Ty2
AE005673	Caulobacter crescentus CB15
AP010935	Streptococcus dysgalactiae subsp. equisimilis GGS˙124
CP002447	Mesorhizobium ciceri biovar biserrulae WSM1271
AP008226	Thermus thermophilus HB8
CP000053	Rickettsia felis URRWXCal2
CP001161	Buchnera aphidicola str. 5A (Acyrthosiphon pisum)
AP012157	Solibacillus silvestris StLB046
CP000568	Clostridium thermocellum ATCC 27405
CP001846	Escherichia coli O55
CP000896	Acholeplasma laidlawii PG-8A
CP002108	Mycoplasma leachii PG50
CP001016	Beijerinckia indica subsp. indica ATCC 9039
CP002544	Odoribacter splanchnicus DSM 20712
AP009240	Escherichia coli SE11
CP002898	Leuconostoc sp. C2
CP001131	Anaeromyxobacter sp. K
CP001896	Allochromatium vinosum DSM 180
FP565176	Xanthomonas albilineans GPE PC73
FQ312042	Streptococcus pneumoniae SPN033038
FQ312045	Streptococcus pneumoniae SPN034156
CP002638	Verrucosispora maris AB-18-032
CP002274	Mycoplasma hyopneumoniae 168
CP001358	Desulfovibrio desulfuricans subsp. desulfuricans str. ATCC 27774
CP000282	Saccharophagus degradans 2-40
FN995097	Neisseria lactamica 020-06
CP001722	Zymomonas mobilis subsp. mobilis NCIMB 11163
CP002858	Flexistipes sinusarabici DSM 4947
AB370334	Lactobacillus brevis
CP002048	Syntrophothermus lipocalidus DSM 12680
CP000863	Acinetobacter baumannii ACICU
CP001019	Coxiella burnetii CbuG˙Q212
CP001889	Chlamydia trachomatis G/11074
CP000673	Clostridium kluyveri DSM 555
CP001859	Acidaminococcus fermentans DSM 20731
CP000820	Frankia sp. EAN1pec
CP002464	Lactobacillus johnsonii DPC 6026
AL935263	Lactobacillus plantarum WCFS1
CP000255	Staphylococcus aureus subsp. aureus USA300˙FPR3757
CP000538	Campylobacter jejuni subsp. jejuni 81-176
CM000754	Bacillus thuringiensis serovar andalousiensis BGSC 4AW1
CP001111	Stenotrophomonas maltophilia R551-3
CP000103	Nitrosospira multiformis ATCC 25196
AE017244	Mycoplasma hyopneumoniae 7448
CP001630	Actinosynnema mirum DSM 43827
CP001392	Acidovorax ebreus TPSY
AM420293	Saccharopolyspora erythraea NRRL 2338
CP000241	Helicobacter pylori HPAG1
CP001706	Jonesia denitrificans DSM 20603
CP002830	Myxococcus fulvus HW-1
CP000159	Salinibacter ruber DSM 13855
CP002487	Salmonella enterica subsp. enterica serovar Typhimurium str. ST4/74
AE014133	Streptococcus mutans UA159
CP001891	Klebsiella variicola At-22
AE006914	Rickettsia conorii str. Malish 7
AE016828	Coxiella burnetii RSA 493
CP000237	Neorickettsia sennetsu str. Miyayama
CP000127	Nitrosococcus oceani ATCC 19707

CP002628	Coriobacterium glomerans PW2
AP012211	Eggerthella sp. YY7918
CP000614	Burkholderia vietnamiensis G4
FN543093	Cronobacter turicensis z3032
CP000249	Frankia sp. CcI3
CP002771	Marinomonas posidonica IVIA-Po-181
CP002094	Lactococcus lactis subsp. cremoris NZ9000
CP000901	Yersinia pestis Angola
CP000552	Prochlorococcus marinus str. MIT 9515
CP002008	Caulobacter segnis ATCC 21756
CP001969	Escherichia coli IHE3034
CP000411	Oenococcus oeni PSU-1
CP000628	Agrobacterium radiobacter K84
CP000133	Rhizobium etli CFN 42
CP001839	Thermotoga naphthophila RKU-10
CM000490	Bacillus subtilis subsp. subtilis str. SMY
FN665654	Clostridium difficile 2007855
FQ311875	Arthrobacter arilaitensis Re117
CU928161	Escherichia coli S88
CP002491	Enterococcus faecalis 62
CP002666	Cellulomonas fimi ATCC 484
CP000937	Francisella philomiragia subsp. philomiragia ATCC 25017
CP000408	Streptococcus suis 98HAH33
AE009442	Xylella fastidiosa Temecula1
CP001964	Cellulomonas flavigena DSM 20109
FP929055	Ruminococcus torques L2-14
CP001601	Corynebacterium aurimucosum ATCC 700975
CP002562	Riemerella anatipestifer RA-GD
CP000425	Lactococcus lactis subsp. cremoris SK11
CP001890	Chlamydia trachomatis E/11023
CP000472	Shewanella piezotolerans WP3
CP002027	synthetic Mycoplasma mycoides JCVI-syn1.0
CP000407	Streptococcus suis 05ZYH33
CP002107	Mycoplasma mycoides subsp. mycoides SC str. Gladysdale
FN597644	Bacillus amyloliquefaciens DSM 7
CP002513	Mycoplasma bovis Hubei-1
CP000362	Roseobacter denitrificans OCh 114
AP009044	Corynebacterium glutamicum R
CP001712	Robiginitalea biformata HTCC2501
AM884176	Chlamydia trachomatis 434/Bu
FN650140	Legionella longbeachae NSW150
CP001715	Candidatus Accumulibacter phosphatis clade IIA str. UW-1
AE005176	Lactococcus lactis subsp. lactis Il1403
FQ312004	Bacteroides fragilis 638R
CP001686	Kytococcus sedentarius DSM 20547
CP001977	Propionibacterium acnes SK137
CP001836	Dickeya dadantii Ech586
AP006725	Klebsiella pneumoniae subsp. pneumoniae NTUH-K2044
CP001802	Gordonia bronchialis DSM 43247
CP000061	Aster yellows witches'-broom phytoplasma AYWB
CP000262	Streptococcus pyogenes MGAS10750
AP010953	Escherichia coli O26
CP002622	Pseudomonas stutzeri DSM 4166
CP001830	Sinorhizobium meliloti SM11
FR773526	Clostridium botulinum H04402 065
CP000139	Bacteroides vulgatus ATCC 8482
AP012053	Streptococcus gallolyticus subsp. gallolyticus ATCC 43143
CP002040	Nocardiopsis dassonvillei subsp. dassonvillei DSM 43111
CP001113	Salmonella enterica subsp. enterica serovar Newport str. SL254
AE014292	Brucella suis 1330
AE014074	Streptococcus pyogenes MGAS315
AM889136	Neisseria meningitidis alpha14
CP002440	Neisseria gonorrhoeae TCDC-NG08107
CP002118	Clostridium acetobutylicum EA 2018
CP002424	Neisseria meningitidis NZ-05/33
FR729477	Yersinia enterocolitica subsp. palearctica Y11
CP000447	Shewanella frigidimarina NCIMB 400

CP000017	Streptococcus pyogenes MGAS5005
FN645454	Bartonella clarridgeiae 73
CP000352	Cupriavidus metallidurans CH34
CP001083	Clostridium botulinum Ba4 str. 657
CP001892	Bifidobacterium animalis subsp. lactis V9
CP000454	Arthrobacter sp. FB24
CP002059	'Nostoc azollae' 0708
CP000510	Psychromonas ingrahamii 37
CP001048	Yersinia pseudotuberculosis PB1/+
BX119912	Rhodopirellula baltica SH 1
CP000494	Bradyrhizobium sp. BTAi1
CP002287	Achromobacter xylosoxidans A8
CM000756	Bacillus thuringiensis serovar huazhongensis BGSC 4BD1
CP001823	Sphaerobacter thermophilus DSM 20745
AP006861	Chlamydophila felis Fe/C-56
CP002663	Pusillimonas sp. T7-7
CP001173	Helicobacter pylori G27
FQ859181	Hyphomicrobium sp. MC1
AE008922	Xanthomonas campestris pv. campestris str. ATCC 33913
CP002379	Arthrobacter phenanthrenivorans Sphe3
CP002383	Shewanella baltica OS678
CP002344	Thermaerobacter marianensis DSM 12885
CU928145	Escherichia coli 55989
AE000783	Borrelia burgdorferi B31
FM252031	Streptococcus suis SC84
CP001098	Halothermothrix orenii H 168
CP000783	Cronobacter sakazakii ATCC BAA-894
CP001393	Caldicellulosiruptor bescii DSM 6725
CP001616	Tolumonas auensis DSM 9187
FM179323	Lactobacillus rhamnosus Lc 705
CP001357	Brachyspira hyodysenteriae WA1
CP002343	Intrasporangium calvum DSM 43043
AE007869	Agrobacterium fabrum str. C58
CP001779	Streptobacillus moniliformis DSM 12112
CP002568	Polymorphum gilvum SL003B-26A1
CP002572	Helicobacter pylori 2018
CP001818	Thermanaerovibrio acidaminovorans DSM 6589
AE017263	Mesoplasma florum L1
CP001758	Leuconostoc kimchii IMSNU 11154
CP002801	Frankia symbiont of Datisca glomerata
CM000658	Clostridium difficile QCD-37x79
CP001828	Legionella pneumophila 2300/99 Alcoy
CP002248	Agrobacterium sp. H13-3
AE017198	Lactobacillus johnsonii NCC 533
CP000626	Vibrio cholerae O395
CP002215	Streptococcus dysgalactiae subsp. equisimilis ATCC 12394
CP002903	Spirochaeta thermophila DSM 6578
AE017180	Geobacter sulfurreducens PCA
CP002246	Yersinia enterocolitica subsp. palearctica 105.5R(r)
AL123456	Mycobacterium tuberculosis H37Rv
AM286415	Yersinia enterocolitica subsp. enterocolitica 8081
FN822744	Leuconostoc gasicomitatum LMG 18811
CP000699	Sphingomonas wittichii RW1
CP001825	Thermobaculum terrenum ATCC BAA-798
CP002631	Treponema succinifaciens DSM 2489
CP001236	Vibrio cholerae O395
CP000514	Marinobacter aquaeolei VT8
AE017245	Mycoplasma synoviae 53
CP002453	Cellulophaga algicola DSM 14237
CP002806	Chlamydophila psittaci 02DC15
AE000520	Treponema pallidum subsp. pallidum str. Nichols
CP002431	Desulfovibrio aespoeensis Aspo-2
CM000716	Bacillus cereus BGSC 6E1
AE000513	Deinococcus radiodurans R1
CP002014	Burkholderia sp. CCGE1002
CP000608	Francisella tularensis subsp. tularensis WY96-3418
CP002207	Bacillus atrophaeus 1942

AE017221	Thermus thermophilus HB27
CP000361	Arcobacter butzleri RM4018
AE002161	Chlamydophila pneumoniae AR39
CP001821	Xylanimonas cellulosilytica DSM 15894
AP009324	Staphylococcus aureus subsp. aureus Mu3
CM000742	Bacillus mycoides DSM 2048
FN869568	Halomonas elongata DSM 2581
CP000560	Bacillus amyloliquefaciens FZB42
AE006468	Salmonella enterica subsp. enterica serovar Typhimurium str. LT2
CP000124	Burkholderia pseudomallei 1710b
CP000686	Roseiflexus sp. RS-1
AL592022	Listeria innocua Clip11262
CP001074	Rhizobium etli CIAT 652
AE000516	Mycobacterium tuberculosis CDC1551
AE006470	Chlorobium tepidum TLS
HE572590	Mycobacterium canettii CIPT 140010059
AM406670	Azoarcus sp. BH72
FN545816	Clostridium difficile R20291
AP006618	Nocardia farcinica IFM 10152
FP929047	Gordonibacter pamelaeae 7-10-1-b
CP001668	Mycoplasma mycoides subsp. capri str. GM12
FP929042	Eubacterium rectale DSM 17629
CP002659	Sphaerochaeta coccoides DSM 17374
AM884177	Chlamydia trachomatis L2b/UCH-1/proctitis
CP000377	Ruegeria sp. TM1040
AM421808	Neisseria meningitidis FAM18
CP000087	Rickettsia bellii RML369-C
AM933172	Salmonella enterica subsp. enterica serovar Enteritidis str. P125109
AL954747	Nitrosomonas europaea ATCC 19718
CP001997	Aminobacterium colombiense DSM 12261
CP002449	Alicycliphilus denitrificans BC
CP001056	Clostridium botulinum B str. Eklund 17B
AM942759	Proteus mirabilis HI4320
CP000542	Verminephrobacter eiseniae EF01-2
CM000733	Bacillus cereus Rock3-44
AP012054	Streptococcus pasteurianus ATCC 43144
CP002360	Mahella australiensis 50-1 BON
CP000908	Methylobacterium extorquens PA1
CP002807	Chlamydophila psittaci 08DC60
AP008955	Brevibacillus brevis NBRC 100599
CP001205	Borrelia burgdorferi ZS7
AE017226	Treponema denticola ATCC 35405
CP002228	Borrelia burgdorferi N40
CP002403	Ruminococcus albus 7
AM746676	Sorangium cellulosum So ce56
CP002479	Geobacter sp. M18
CP001130	Hydrogenobaculum sp. Y04AAS1
AL590842	Yersinia pestis CO92
BK006741	Francisella tularensis subsp. holarctica OSU18
CP000387	Streptococcus sanguinis SK36
CP000153	Sulfurimonas denitrificans DSM 1251
AE014075	Escherichia coli CFT073
CM000715	Bacillus cereus ATCC 10876
CP000056	Streptococcus pyogenes MGAS6180
CP000792	Campylobacter concisus 13826
CP000698	Geobacter uraniireducens Rf4
CP001114	Deinococcus deserti VCD115
CP001608	Yersinia pestis biovar Medievalis str. Harbin 35
AB370337	Lactobacillus brevis
CP001047	Mycoplasma arthritidis 158L3-1
CP000227	Bacillus cereus Q1
CP000744	Pseudomonas aeruginosa PA7
AP009247	Candidatus Vesicomyosocius okutanii HA
CP000927	Caulobacter sp. K31
AE005672	Streptococcus pneumoniae TIGR4
CP000826	Serratia proteamaculans 568
AL157959	Neisseria meningitidis Z2491

CP001177	Bacillus cereus AH187
CP002184	Helicobacter pylori 908
BA000019	Nostoc sp. PCC 7120
DS995265	Leptospirillum sp. Group II '5-way CG'
CM000775	Burkholderia pseudomallei 1106b
CP000909	Chloroflexus aurantiacus J-10-fl
FR714927	Staphylococcus aureus subsp. aureus ECT-R 2
CM000912	Aggregatibacter actinomycetemcomitans D7S-1
AB370335	Lactobacillus brevis
AE016826	Buchnera aphidicola str. Bp (Baizongia pistaciae)
CP002541	Sphaerochaeta globus str. Buddy
CP001918	Enterobacter cloacae subsp. cloacae ATCC 13047
FR687359	Burkholderia rhizoxinica HKI 454
AM933173	Salmonella enterica subsp. enterica serovar Gallinarum str. 287/91
CP000267	Rhodoferax ferrireducens T118
CP002417	Variovorax paradoxus EPS
CP000539	Acidovorax sp. JS42
CP002244	Candidatus Tremblaya princeps PCIT
CP000573	Burkholderia pseudomallei 1106a
CP002585	Pseudomonas brassicacearum subsp. brassicacearum NFM421
BX548175	Prochlorococcus marinus str. MIT 9313
CP001993	Streptococcus pneumoniae TCH8431/19A
CP002105	Acetohalobium arabaticum DSM 5501
CP002729	Escherichia coli UMNK88
CR626927	Bacteroides fragilis NCTC 9343
AB370336	Lactobacillus brevis
AP010803	Sphingobium japonicum UT26S
CT009589	Corynebacterium glutamicum
AM942444	Corynebacterium urealyticum DSM 7109
AE008917	Brucella melitensis bv. 1 str. 16M
CP002559	Lactobacillus acidophilus 30SC
CP002563	Carnobacterium sp. 17-4
AP009256	Bifidobacterium adolescentis ATCC 15703
FN668375	Clostridium difficile M68
FN652779	Chlamydia trachomatis Sweden2
CP001101	Chlorobium phaeobacteroides BS1
FN665652	Clostridium difficile CF5
CP002364	Desulfobulbus propionicus DSM 2032
AP012204	Microlunatus phosphovorus NM-1
CP000100	Synechococcus elongatus PCC 7942
AE016824	Leptospira interrogans serovar Copenhageni str. Fiocruz L1-130
CP000141	Carboxydothermus hydrogenoformans Z-2901
FP236843	Erwinia billingiae Eb661
FR872582	Simkania negevensis Z
CP001750	Bifidobacterium dentium Bd1
CM000854	Campylobacter jejuni subsp. jejuni 1336
CP000802	Escherichia coli HS
CM000739	Bacillus cereus AH1271
AM295250	Staphylococcus carnosus subsp. carnosus TM300
CP002377	Vibrio furnissii NCTC 11218
AP009552	Microcystis aeruginosa NIES-843
CU633749	Cupriavidus taiwanensis LMG 19424
CP000800	Escherichia coli E24377A
CP002175	Halanaerobium praevalens DSM 2228
FP929032	Alistipes shahii WAL 8301
CP002418	Rhodopseudomonas palustris DX-1
FN649414	Escherichia coli ETEC H10407
CP001515	Bifidobacterium animalis subsp. lactis Bl-04
CP001107	Eubacterium rectale ATCC 33656
CP000806	Cyanothece sp. ATCC 51142
CP001154	Laribacter hongkongensis HLHK9
FN598874	Helicobacter pylori B8
CP000551	Prochlorococcus marinus str. AS9601
AL513382	Salmonella enterica subsp. enterica serovar Typhi str. CT18
CP002410	Clostridium botulinum BKT015925
CP000094	Pseudomonas fluorescens Pf0-1
CP002031	Geobacter sulfurreducens KN400

CP002912	Rickettsia heilongjiangensis 054
AP009178	Nitratiruptor sp. SB155-2
CP002740	Sinorhizobium meliloti BL225C
CR848038	Chlamydophila abortus S26/3
CU928158	Escherichia fergusonii ATCC 35469
CP000872	Brucella canis ATCC 23365
CP001312	Rhodobacter capsulatus SB 1003
FR687201	Legionella pneumophila 130b
CP000697	Acidiphilium cryptum JF-5
U00089	Mycoplasma pneumoniae M129
CP002475	Streptomyces flavogriseus ATCC 33331
CU928164	Escherichia coli IAI39
CP000003	Streptococcus pyogenes MGAS10394
CP002633	Streptococcus suis ST3
CP002220	Bifidobacterium bifidum S17
AP008937	Lactobacillus fermentum IFO 3956
CP001842	cyanobacterium UCYN-A
CP002695	Bordetella pertussis CS
CP000266	Shigella flexneri 5 str. 8401
CP002915	Bifidobacterium animalis subsp. lactis CNCM I-2494
CM000734	Bacillus cereus Rock4-2
FP929037	Clostridium cf. saccharolyticum K10
CP002530	Bacteroides salanitronis DSM 18170
CP001614	Teredinibacter turnerae T7901
CM000759	Bacillus thuringiensis IBL 4222
CP002897	Paracoccus denitrificans SD1
CP000903	Bacillus weihenstephanensis KBAB4
CP001673	Flavobacteriaceae bacterium 3519-10
CP000058	Pseudomonas syringae pv. phaseolicola 1448A
CP001429	Blattabacterium sp. (Periplaneta americana) str. BPLAN
AE014073	Shigella flexneri 2a str. 2457T
CP000002	Bacillus licheniformis DSM 13 = ATCC 14580
AE001437	Clostridium acetobutylicum ATCC 824
AL591824	Listeria monocytogenes EGD-e
FQ312006	Haemophilus influenzae 10810
FP671138	Mycoplasma agalactiae
FR875178	Streptococcus thermophilus JIM 8232
AP010918	Mycobacterium bovis BCG str. Tokyo 172
CP001582	Helicobacter pylori v225d
CP001876	Campylobacter jejuni subsp. jejuni IA3902
AM236080	Rhizobium leguminosarum bv. viciae 3841
CM000727	Bacillus cereus 95/8201
CP001001	Methylobacterium radiotolerans JCM 2831
AE015450	Mycoplasma gallisepticum str. R(low)
FM180568	Escherichia coli O127
CP002304	Halanaerobium hydrogeniformans
CP001657	Pectobacterium carotovorum subsp. carotovorum PC1
AP012029	Anaerolinea thermophila UNI-1
CP000647	Klebsiella pneumoniae subsp. pneumoniae MGH 78578
CP000854	Mycobacterium marinum M
AE014295	Bifidobacterium longum NCC2705
CP000351	Leptospira borgpetersenii serovar Hardjo-bovis str. JB197
AP009378	Escherichia coli SE15
AL009126	Bacillus subtilis subsp. subtilis str. 168
AP011529	Deferribacter desulfuricans SSM1
AP008231	Synechococcus elongatus PCC 6301
CP002584	Sphingobacterium sp. 21
CP002790	Corynebacterium ulcerans 809
CP000548	Burkholderia mallei NCTC 10247
CP002109	Clostridium saccharolyticum WM1
CP001579	Brucella microti CCM 4915
CP002029	Campylobacter jejuni subsp. jejuni ICDCCJ07001
CP001982	Bacillus megaterium DSM 319
CP001620	Corynebacterium kroppenstedtii DSM 44385
FM991728	Helicobacter pylori B38
CP000768	Campylobacter jejuni subsp. doylei 269.97
CP000148	Geobacter metallireducens GS-15

AE008918	Brucella melitensis bv. 1 str. 16M
CP000155	Hahella chejuensis KCTC 2396
FM872308	Chlamydia trachomatis B/Jali20/OT
CP002177	Acinetobacter calcoaceticus PHEA-2
AE001363	Chlamydophila pneumoniae CWL029
CP002056	Methylotenera versatilis 301
CP000919	Streptococcus pneumoniae JJA
CP001888	Chlamydia trachomatis G/11222
CU928162	Escherichia coli ED1a
CP001713	Chlamydophila pneumoniae LPCoLN
CP002432	Desulfurispirillum indicum S5
AE015929	Staphylococcus epidermidis ATCC 12228
CP002420	Neisseria meningitidis H44/76
CP000021	Vibrio fischeri ES114
CP000029	Staphylococcus epidermidis RP62A
CP001699	Chitinophaga pinensis DSM 2588
FP885897	Ralstonia solanacearum CFBP2957
CP001618	Beutenbergia cavernae DSM 12333
AE017355	Bacillus thuringiensis serovar konkukian str. 97-27
CP001390	Geobacter daltonii FRC-32
CP001383	Shigella flexneri 2002017
CP001050	Neisseria gonorrhoeae NCCP11945
CP000947	Haemophilus somnus 2336

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