

Molecular Methods for Monitoring Bacterial Gene Expression During Infection

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TABLE OF CONTENTS

1. INTRODUCTION	3
2. THE REVOLUTIONARY TALE OF <i>IVI</i> GENE IDENTIFICATION	4
2.1. POSITIVE SELECTION <i>IN VIVO</i>	4
2.1.1. <i>IVET</i>	4
2.1.2. <i>Differential Fluorescence Induction (DFI)</i>	6
2.2. NEGATIVE SELECTION <i>IN VIVO</i> ; SIGNATURE TAGGED MUTAGENESIS.....	6
2.3. DIRECT DETECTION OF <i>IVI</i> GENE EXPRESSION.....	8
2.3.1. <i>Subtractive Hybridisation</i>	8
2.3.2. <i>Differential Display</i>	9
2.3.3. <i>Limitations of Subtractive Hybridisation and Differential Display and possible alternatives</i>	10
2.3.4. <i>Proteomics</i>	12
2.4. <i>IN VIVO</i> INDUCED ANTIGEN TECHNOLOGY.....	13
3. THE CHALLENGE OF MONITORING GENE EXPRESSION IN INDIVIDUAL CELLS	14
3.1. <i>IN SITU</i> PCR.....	14
3.2. TRANSCRIPTIONAL REPORTER SYSTEMS.....	14
3.2.1. <i>β-Galactosidase</i>	14
3.2.2. <i>Luciferases</i>	15
3.2.3. <i>Green Fluorescent Protein</i>	16
4. FUTURE PROSPECTS: UNDERSTANDING THE COMPLEX DIALOG BETWEEN THE HOST AND THE PATHOGEN	18
4.1. MICROSCOPY.....	18
4.2. FLOW CYTOMETRY.....	20
4.3. GLOBAL EXPRESSION PROFILING.....	21
4.4. TISSUE CULTURE MODELS.....	22
4.5. ANIMAL MODELS.....	25
5. CONCLUSION	28
REFERENCES	30

1. Introduction

Bacterial infections involve a myriad of bacterial-encoded virulence determinants, which are produced in response to the host cellular environment. The expression cascade of virulence factors is likely to occur in the appropriate place, at the appropriate stage of infection and at the appropriate level to achieve successful infection by the pathogen. A number of bacterial virulence factors have been shown to alter normal host gene expression, leading to production of new host environmental signals which in turn can cause further bacterial adaptation. Despite our increasing knowledge relating to bacterial virulence and host response determinants, this cross-talk mechanism remains unclear. It has become necessary to develop novel techniques for i) identifying determinants involved in bacterial virulence, as well as their inducing signals; ii) measuring their expression levels during infection; iii) understanding the interactions of host and bacterial factors. *In vivo* expression Technology, Differential Fluorescence Induction and transposon-based approaches have given new insights into bacterial virulence. The increasing availability of genome sequences, of improved reporter systems, the development of new technologies such as *in situ* RT-PCR, DNA Microarray technology, as well as the combination of several physical techniques (for example *in situ* hybridisation and flow cytometry) promise to provide a clearer understanding of bacterial pathogenicity and to contribute to the development of new anti-microbial agents and vaccines.

In this chapter we aim to give a brief description of currently available techniques used to identify *in vivo* induced (*ivi*) genes and to monitor bacterial gene expression during infection. We also comment on new approaches currently being developed to clarify important aspects of the host/pathogen interaction. For the purposes of this review “*in vivo*” refers to studies involving an animal model, and “*in vitro*” refers to experiments in cultured cells or in laboratory media.

2. The revolutionary tale of *ivi* gene identification

The fact that much virulence gene expression is usually environmentally induced has now been clearly shown for a large number of microbial pathogens. However, researchers have had to rely on *in vitro* systems as a crude representation of the dynamic milieu of the host cell or the complexity of an entire animal. The environment of the mammalian cell is constantly reacting to different signals, and many levels of cellular response contribute to the global homeostatic status of the host. Therefore, the limited numbers of bacterial genes identified as simply responding to environmental signals experienced in *in vitro* systems do not reflect the complexities of a living host. These limitations have been overcome in the last decade by the application of new technologies such as *In Vivo* Expression Technology (IVET), Differential Fluorescence Induction (DFI) and Signature Tagged Mutagenesis (STM).

2.1. POSITIVE SELECTION *IN VIVO*

2.1.1. IVET

In Vivo Expression Technology is based on a stringent selection system for identifying genes which are switched on *in vivo*. It involves the random insertion of chromosomal DNA fragments upstream of a promoter-less reporter gene (such as *purA::lacZY*, *cat*, *tnpR*) whose expression allows the survival of the bacteria in an animal model. Only the bacteria that express the reporter gene under the control of an *ivi* gene promoter will survive this procedure (Heithoff *et al.*, 1997; Mahan *et al.*, 1995; Merrell and Camilli, 2000).

IVET has allowed the identification of hundreds of *ivi* genes in a diverse range of bacterial pathogens including *Salmonella enterica* sv. Typhimurium (Mahan *et al.*, 1993), *Pseudomonas aeruginosa* (Wang *et al.*, 1996), *Staphylococcus aureus* (Lammers *et al.*, 2000; Lowe *et al.*, 1998), *Vibrio cholerae* (Camilli *et al.*, 1994) and *Candida albicans* (Staib *et al.*,

1999) (Table 1). IVET applications have not been limited to animal models, but have also involved cultivated cells for pathogens such as *S. typhi* (Staendner *et al.*, 1995) and *S. Typhimurium* (Janakiraman and Schlauch, 2000). Because IVET can be used in different models for the same pathogen, *ivi* genes which respond to distinct environmental conditions have been identified (e.g. *P. aeruginosa* (See Table 1)).

Many *ivi* genes have been characterised in great detail, and include a mixture of genes of known function (for example *pvdI*, *soxR*, *ppkA* in *P. aeruginosa* (Ha and Jin, 1999; Handfield *et al.*, 2000b; Motley and Lory, 1999) with genes of unknown function [FUN genes (Hinton, 1997)]. A clear and well illustrated description of IVET and the various classes of genes identified by IVET has recently been presented (Merrell and Camilli, 2000).

The IVET approach has evolved a great deal from the original *purA* and *cat*-based selection systems. A selection, based on transcriptional fusions of *ivi* promoters with the *tnpR* recombinase gene, has allowed identification of *ivi* genes expressed transiently or at a low-level (Camilli *et al.*, 1994; Camilli and Mekalanos, 1995; Merrell and Camilli, 2000). This adaptation of IVET has been named RIVET, **R**ecombinase-based **I**VET (Lee and Camilli, 2000; Schlauch and Camilli, 2000).

However, the IVET/RIVET technique possesses a number of limitations. First, it requires the development of genetic tools, restricting the number of applicable pathogens. Second, the selective system itself presents inherent limitations such as the need for an auxotrophic strain (e.g. *purA* mutant), the administration of antibiotic solutions at the appropriate concentrations (e.g. *cat*) or the over-sensitivity of the *tnpR*-based system in some model systems. A tunable RIVET approach has recently been developed to allow the identification of *ivi* genes which show a significant basal expression level *in vitro* that would have been missed with the original technique (Merrell and Camilli, 2000). This is based on

various mutations introduced to the RBS of the recombinase to offer a large choice of reporter systems with varying sensitivity and different thresholds for *ivi* gene selection.

2.1.2. Differential Fluorescence Induction (DFI)

As an alternative to the IVET-based promoter-trap systems, a complementary approach has been developed for *ivi* gene identification. **Differential Fluorescence Induction (DFI)** relies on the expression of the enhanced Green Fluorescent Protein (GFP) as a reporter of promoter activity. GFP derives from the jellyfish, *Aequorea victoria*, and will be described later in more detail (See section 3.2.3.). It does not require any substrate or co-factor to fluoresce and thus can be used to monitor gene expression in living samples. DFI involves the cloning of random fusions of genomic DNA fragments upstream of a promoter-less *gfp* gene. Bacteria harbouring *gfp* fusions are pooled and either used to infect cultured mammalian cells (Valdivia and Falkow, 1997), or submitted to various stimuli, such as low pH (Valdivia and Falkow, 1996). Fluorescence Activated Cell Sorting (FACS) is then used to enrich for green fluorescent mammalian cells allowing isolation of GFP-expressing bacteria. This technique can be applied to more complex environments including host tissue ((Valdivia and Ramakrishnan, 2000) and the IVIF technique described in section 4.5). However, DFI, like IVET, will not identify genes which are expressed *in vitro* and are also important for virulence *in vivo*. Further experiments are always needed to determine whether *ivi* genes are crucial for bacterial virulence and survival in the host.

2.2. NEGATIVE SELECTION IN VIVO; SIGNATURE TAGGED MUTAGENESIS

Gene inactivation by transposon insertion has been extensively used to assess the role of genes of interest on particular bacterial functions (Kleckner *et al.*, 1977). However, screening of individual mutants to identify genes implicated in host survival or virulence is time

consuming when mutants are screened individually. A new technique based on transposon mutagenesis has been developed to overcome this limitation, Signature Tagged Mutagenesis (STM).

STM allows the identification of genes required for *in vivo* virulence and survival of the pathogen, based on negative selection system (Lehoux and Levesque, 2000; Lehoux *et al.*, 1999; Shea *et al.*, 2000; Unsworth and Holden, 2000). The technique involves comparative hybridisation following efficient random transposition or use of other insertional genetic tools. Different small DNA sequences are used to mark individual transposons so that each insertion harbours a specific signature tag. This allows the testing of a large number of different mutants at the same time, each mutant being distinguishable by its unique tag sequence (Hensel, 1998).

STM was used for the first time in *S. Typhimurium* (Hensel *et al.*, 1995). A mutant library was generated and tested in mice after intraperitoneal injection of pools of 96 mutants. PCR reactions were performed on DNA extracted from bacteria recovered from spleen and products were used as new probes to be tested on the initial library. The absence of signal revealed mutations that were lethal to the bacteria. This technique allowed identification of the second pathogenicity island (SPI2) of *S. Typhimurium*, which is required for systemic spread and intracellular survival of the pathogen (Cirillo *et al.*, 1998; Shea *et al.*, 1996, Shea, 1999 #454). Since then, many virulence genes have been identified by STM in various pathogens, (Table 1). The related GAMBIT (Genomic Analysis and Mapping by *In vitro* Transposition) allows identification of essential genes *in vitro* and is now being used to screen for genes required for survival *in vivo* in animal models (Chiang *et al.*, 1999).

In an analogous fashion to IVET, the same STM mutant library can also be tested simultaneously in different selective systems (animal model versus cultured cells, or in different animal models). Recently, host-specific virulence determinants were identified by STM in

S. Dublin after challenge in calves and in mice (Bispham *et al.*, 2001). Although STM does not allow the direct monitoring of the expression of virulence genes during infection, it is ideal for identifying candidate *ivi* genes.

The powerful STM technology is subjected to several limitations that include: i) the requirement of a random transposition system, ii) the need to optimise a large number of parameters (the tagging strategy; the complexity and dose of the inocula depending on the administration route; the duration of infection), iii) the problem of polar effects preventing discrimination between single gene or operon insertions. *In vitro* transposition systems could improve targeting the entire genome of the bacterial pathogen. STM has the significant advantage over IVET and DFI of immediately showing the importance of *ivi* genes for virulence and survival rather than just identifying genes that are switched on *in vivo*.

2.3. DIRECT DETECTION OF *IVI* GENE EXPRESSION

The IVET, DFI and STM approaches are very powerful, but are not relevant to genetically-intractable pathogens. Furthermore, these techniques tend to give a “yes or no” answer, rather than precisely measuring levels of gene expression. Therefore alternative techniques have been developed to detect *ivi* messenger RNA directly. Subtractive Hybridisation and Differential Display detect transcripts that are induced *in vivo* and circumvent the need for genetic tools.

2.3.1. Subtractive Hybridisation

Initially developed for eukaryotic research (Kurvari *et al.*, 1995), Subtractive Hybridisation has subsequently been used to study bacterial pathogens. This approach is based on the isolation of mRNA from bacteria grown under conditions of interest (infected mammalian cells or animal models) and the use of reverse transcriptase to generate cDNA. The

cDNA representing the same bacteria grown in broth is used to subtract constitutively-expressed genes by hybridisation. The remaining non-hybridised cDNA is used to identify *ivi* genes for the pathogen under study. Subtractive Hybridisation analysis has proved to be useful in *Mycobacterium avium* during macrophage infection (Plum and Clark-Curtiss, 1994) as well as in several other organisms (Table 1), and is now commonly used to compare genomes between sequenced strains and other strains of interest, allowing identification of differences between pathogens of the same genus that reflect variations in tissue tropism (Dasgupta *et al.*, 2000; Jaufeerally-Fakim *et al.*, 2000; Tinsley and Nassif, 1996; Zhang *et al.*, 2000).

However, several limitations have been found with this approach (Hautefort and Hinton, 2000): First, only two organisms or two gene expression patterns can be compared at once. Second, this technique is hampered by the instability of bacterial mRNA as well as the requirement for high-quality mRNA isolated from small populations of bacteria grown *in vivo*. Finally, transiently-expressed genes may not be well represented; RT-PCR-based amplification can improve the sensitivity of this approach.

2.3.2. Differential Display

Like Subtractive Hybridisation, this technique was initially developed for eukaryotic studies (Liang and Pardee, 1992). It is based on the electrophoretic comparison of mRNA profiles of the same organism grown under various environmental and control conditions. Microbial mRNA is extracted from the organism, reverse-transcribed, PCR-amplified and radio-labelled for auto-radiographic analysis. Differential Display has identified host genes induced by infection of a pathogen such as the macrophage response caused by *M. tuberculosis* infection, the gastric cellular response to *Helicobacter pylori* infection or the mucosal defences

associated with the gut microflora (Ogawa *et al.*, 2000; Ragno *et al.*, 1997; Ragno *et al.*, 1998; Wong *et al.*, 1996).

The lack of polyadenylation of prokaryotic mRNA necessitated modifications of Differential Display for bacterial applications. These involved the use of random primers for reverse transcription and cDNA amplification, and were used for the first time to identify genes of *Legionella pneumophila* that were induced during macrophage infection (Abu Kwaik and Pederson, 1996). Since then Differential Display has been considerably improved (Fleming *et al.*, 1998), leading to the identification of important *ivi* genes in different bacteria (Table 1). Northern blotting and total RNA dot blotting are often combined with Differential Display to confirm variations in transcript levels (Gill *et al.*, 1999; Ragno *et al.*, 1998).

Differential Display has several advantages compared with Subtractive Hybridisation: i) it allows the comparison of more than two different conditions at the same time. ii) Because of the cDNA PCR amplification step, high levels of gene expression are not required, allowing identification of less abundant microbial mRNA with a detection level of at least 10^3 transcript molecules (Fleming *et al.*, 1998). iii) Unlike IVET and STM, Differential Display can be used to study both up- and down-regulation of gene expression.

2.3.3. Limitations of Subtractive Hybridisation and Differential Display and possible alternatives

Subtractive Hybridisation and Differential Display do not show whether expression of particular genes is required for a particular aspect of virulence. The exact role of identified genes must be determined subsequently by genetic mutation and virulence studies in appropriate model systems. The analysis of prokaryotic mRNA expression with Differential Display is known to generate false positives, making it very labour-intensive. The efficiency of

Differential Display is impeded by the short half-life of prokaryotic mRNA, the absence of RNA polyadenylation and still lacks the sensitivity to detect rare mRNA species.

Alternative approaches for the analysis of bacterial mRNA induced *in vivo* have been described. First, Subtractive Cloning is an updated Subtractive Hybridisation method for comparing bacterial gene expression (Carulli *et al.*, 1998; Diatchenko *et al.*, 1999; Sturtevant, 2000). Messenger RNA is reverse-transcribed into cDNA for each condition tested, using different linkers each time. A series of Subtractive Hybridisation experiments allow enrichment of *ivi* cDNA that possess a linker sequence at both ends, allowing direct cloning and sequencing of the identified *ivi* gene.

Serial Analysis of Gene Expression (SAGE) is another useful alternative to Differential Display that can be used to analyse and simultaneously to quantify a large number of transcripts (Velculescu *et al.*, 1995). To demonstrate this strategy, the authors chose a eukaryotic example, pancreatic gene expression. SAGE involves the extraction of tissue mRNA and reverse transcription to cDNA with a biotinylated oligo(dT) primer. Action of appropriate restriction enzymes generates small sequence tags specific to each cDNA molecule, and these are ligated to linker sequences. Compatible linkers allow the concatenation of the different tags and their direct cloning and sequencing. The abundance of each tag reflects the initial proportion of the transcript in the initial mRNA pool. SAGE has been successfully applied to eukaryotes but in principle could also be used in prokaryotes.

Another new hybridisation-based method has been developed to compare gene induction during infection of cell culture or animal tissue models (Graham and Clark-Curtiss, 1999). This technique is called Selective Capture Of Transcribed Sequences (SCOTS), and derives from the initial Subtractive Hybridisation approach used in *M. avium* (Plum and Clark-Curtiss, 1994). SCOTS involves two important steps: The first one aims to normalise levels of

microbial cDNA obtained from bacteria released from different experimental conditions such as infected host cells or animal tissue. This procedure involves an enrichment step for low abundance transcripts. The second step is based on the comparison of gene expression patterns between two environments. Subtractive Hybridisation of normalised cDNA obtained from bacteria grown in the different environments under study reveals preferential expression of genes under one of the other conditions tested. SCOTS has identified numerous macrophage-induced genes in *M. tuberculosis*. Since then it has been used in other pathogens like *S. Typhimurium* allowing identification of a novel fimbrial operon and putative transcriptional regulator that are absent from the *S. typhi* genome (Morrow *et al.*, 1999). Although SCOTS has the advantage of identifying low abundance mRNA, it cannot be used to quantify *in vivo* gene expression levels because of the cDNA normalisation step included in the protocol.

2.3.4. Proteomics

Direct analysis of bacterial gene expression during infection can only reveal events, which have occurred during transcription. Because many levels of gene regulation are post-transcriptional, and contribute directly to translational efficiency it is important to consider expression at the protein level. The obvious goal is to characterise the bacterial proteome during infection, as it genuinely represents the whole bacterial response to the host. Unfortunately, it has not yet been possible to analyse the proteomes of bacteria removed directly from the host, due to a combination of factors including the relatively low numbers of bacteria that can be isolated after infection and the lack of a system for protein amplification. Nevertheless, proteomic technology has been used to detect bacterial proteins induced during infection of cultured cells. This approach relies on the specific labelling of bacterial-encoded proteins expressed within mammalian cells, and has been reviewed elsewhere (Cash, 2000; Hautefort and Hinton, 2000). One particular report on the regulation of the *S. Typhimurium*

SPI2 locus demonstrates the direction that *in vivo* expression research is likely to take in the near future (Deiwick and Hensel, 1999). They used a combination of reporter genes, regulatory mutants and two-dimensional gel electrophoresis to simultaneously characterise SPI2 expression at the level of the transcriptome and the proteome. Once this approach has been combined with identification of the *in vivo*-induced proteome, it will reveal the role of particular regulators in controlling the expression of IVI proteins.

2.4. *IN VIVO* INDUCED ANTIGEN TECHNOLOGY

An innovative approach has been developed to identify IVI proteins expressed during human infection, that does not rely on *in vitro* or animal model systems. The IVIAT approach (*In Vivo* Induced Antigen Technology) is based on the immunological screening of an expression library of the pathogen under study with various sera obtained from human patients infected by this organism (Handfield *et al.*, 2000b). Each batch of sera is previously depleted of antibodies raised against constitutively-expressed proteins using whole bacterial cells and total protein extracts. The straightforward techniques used in IVIAT make it applicable to all type of eukaryotic or prokaryotic pathogens. The most attractive aspect of IVIAT is that it uses genuine human infection samples. IVIAT has successfully identified IVI proteins of *Actinobacillus actinomyetemcomitans* in localized juvenile periodontitis (Handfield *et al.*, 2000a) and is currently being used to study other human pathogens such as *P. aeruginosa* and *Candida* spp. (Hautefort and Hinton, 2000). However, IVIAT requires identification of patients suffering from infection by the pathogen of interest as well as knowing at which stage of infection the sample has been obtained necessitating effective medical involvement.

3. The challenge of monitoring gene expression in individual cells

The ability of bacteria to control and to optimise their growth rate and gene activity by carefully adjusting the cellular composition of DNA, RNA and proteins in response to the prevailing environmental conditions has led to the development of various technical approaches to study gene expression in individual bacterial cells.

3.1. *IN SITU* PCR

This technology has been more widely used to monitor gene expression in eukaryotic than bacterial systems. The use of *in situ* PCR to study *lac* gene expression in *Salmonella* established the sensitivity of the technique in bacteria (Tolker-Nielsen *et al.*, 1997). More recently, the same group have used *in situ* RT-PCR to visualise expression of *dnaK* in *Methanosarcina mazei* (Lange *et al.*, 2000). The combination of *in situ* PCR with flow cytometry offers the potential of selecting individuals from a bacterial population that are expressing particular genes to certain levels (Chen *et al.*, 2000). A wide range of molecular tools can be used to monitor bacterial growth activity *in situ*, has been well reviewed (Molin and Givskov, 1999).

3.2. TRANSCRIPTIONAL REPORTER SYSTEMS

3.2.1. β -Galactosidase

The importance of reporter gene systems for measuring bacterial gene expression *in vivo* has already been described (Hautefort and Hinton, 2000). β -Galactosidase has been used for decades, and has proved to be a reliable screening and reporter system (Jacob and Monod, 1961). It remains very useful for studying up- and down-regulation of gene expression, as well as translational regulation. A practical guide to the construction and use of *lac* fusions in *E. coli*

has been recently published (Hand and Silhavy, 2000). β -Galactosidase is occasionally used for the analysis of gene expression *in vivo* (Hautefort and Hinton, 2000).

The development of new substrates, including fluorescence-generating substrates has considerably improved the sensitivity of β -Galactosidase detection, and has facilitated studies with intact individual bacterial cells (Zhang *et al.*, 1991). The use of β -galactosidase with the Fluorescein di- β -D-galactopyranoside (FDG) substrate was compared to the use of the Green Fluorescent Protein (GFP) for monitoring *gyrB* expression in *Mycobacterium* species (Rowland *et al.*, 1999). Detection of β -Galactosidase with FDG was 70-fold more sensitive than GFP in *M. bovis* BCG when measured by fluorimetry, and comparable to GFP when measured by flow cytometry in individual bacterial cells. However, this study did not use the latest brightly fluorescent derivatives of GFP which are likely to prove more sensitive than β -Galactosidase, even with FDG as a substrate. Furthermore, permeabilisation of bacterial cells is required to deliver β -Galactosidase substrate; due to variations in permeability, the amount of substrate that enters bacterial cells varies from one cell to another, preventing the accurate measurement of changes in gene expression levels in individual bacteria (Nwoguh *et al.*, 1995).

3.2.2. Luciferases

Luciferase catalyses production of photons, and can be used as a reporter of gene expression in appropriate situations. The short half-life of luciferase guarantees real time observation of gene expression. In *Yersinia pseudotuberculosis*, *luxAB* fusions showed that *yopE* and *yopH* expression was highest during early stages of colonisation of either Peyer's patches or the spleen of infected mice (Forsberg and Rosqvist, 1993). Expression of the Lux system derived from *Photobacterium* has been used to follow the real time trafficking of pathogens, such as *S. Typhimurium* or *Staphylococcus aureus* in a live mouse (Contag *et al.*, 1995; Francis *et al.*, 2000). However, the use of luciferase for monitoring gene expression *in*

in vivo has not yet been reported, due to the following problems. First, luciferase is sensitive to the concentration of molecular oxygen. An example of the unreliability of luciferase and *lux* gene fusions to monitor gene expression was shown by the discovery that a *plac::luxAB* fusion in *Salmonella* exhibited a 10-fold reduction in luciferase expression immediately following invasion of epithelial cells. Similar results were observed following contact with tissue culture media, and probably reflect decreased oxygen tension (Maurer *et al.*, 2000). Second, detection of luciferase bioluminescence has not been improved enough to allow measurement of gene expression in single bacterial cells. Finally, fusion of firefly-derived luciferase to eukaryotic gene promoters for monitoring induction upon bacterial invasion produced data that was not consistent with results obtained with β -galactosidase or *cat* reporter systems (Savkovic *et al.*, 2000). The authors observed that bacteria such as EPEC and *S. Typhimurium* that express intact type-III secretion machinery are responsible for the decrease of *luc* activity. Consequently, the use of luciferase to monitor bacterial virulence gene expression has not been widespread.

3.2.3. Green Fluorescent Protein

The Green Fluorescent Protein (GFP) offers the advantage of being naturally fluorescent, without the addition of exogenous substrates. Because the fluorescence of GFP is linearly proportional to the amount of GFP protein, it is an attractive reporter for monitoring *in situ* gene expression in single cells. GFP was used as a reporter system for the first time in eukaryotes in 1994 (Chalfie *et al.*, 1994). Its adaptation for microbial studies led to the development of optimised, more soluble, brighter, blue- or red-shifted mutants extending potential applications (Cormack *et al.*, 1996; Crameri *et al.*, 1996; Heim *et al.*, 1994; Siemering *et al.*, 1996). GFP has now been successfully used to monitor pathogen trafficking within host tissue (Errampalli *et al.*, 1999; Kohler *et al.*, 2000).

However, the major limitation of many GFP mutant proteins is their inability to fold correctly. For example, only 20% of GFPmut1 is folded properly and is able to fluoresce (Tsien, 1998). In 1997 an improved GFP cloning cassette was developed for the generation of prokaryotic transcriptional fusions. It contains a *gfp* gene expressing the S65T “red shift” and the F64L “protein solubility” amino acid substitutions (Heim *et al.*, 1995; Cormack *et al.*, 1996) flanked by convenient restriction sites, a translational enhancer and a consensus ribosome-binding site with an optimised spacer region (Miller and Lindow, 1997). The authors observed a 40- to 80-fold brighter fluorescence with these *gfp* fusions than with fusions to wild-type GFP. Recently an even brighter GFP protein (GFP⁺) has been developed (Scholz *et al.*, 2000). GFP⁺ combines the mutations from GFPuv, which confer enhanced folding, and the mutation from GFPmut1 to give a protein with 130-fold brighter signal than wild type GFP. Various sets of GFP-based vectors have been constructed for Gram-negative bacteria other than *E. coli*. For example, one of these vectors facilitates the generation of GFP transcription fusions in *E. coli* for subsequent introduction into the final host strain, whereas another allows the direct construction of insertions in the host strain (Matthysse *et al.*, 1996). Recent “promoter-trap” vectors have been developed using *gfp* or *inaZ* as reporter genes (Miller *et al.*, 2000). These vectors are claimed to be highly stable in a broad-range of bacterial species and also to encode GFP stability variants with different half-lives, allowing the study of gene repression as well as gene induction.

The use of *gfp* as a reporter of promoter activity has had one notable limitation. A recent comparison was made between the *luxAB* reporter genes from *Vibrio* spp. and the *gfp* reporter system developed by Cramer *et al.* (1996). Both were used in promoter-trap vectors in the cyanobacterium *Synechocystis* sp. PCC6803 (Kunert *et al.*, 2000). For both *isiAB::gfp* and *isiAB::luxAB* fusions, the induction rate of mRNA synthesis was shown to be identical. However, the *gfp* fusion showed a slow increase and decrease of fluorescence whereas the

luxAB fusion caused a rapid increase and decrease in luminescence, probably due to the time taken for formation of active fluorophore from GFP protein. Therefore, the use of GFP has not been ideal for real time experiments that involve rapid changes in gene expression. Enhanced versions of GFP are known to become fluorescent much faster than wild-type GFP, which should improve applicability of GFP (Cormack *et al.*, 1996)

4. Future prospects: Understanding the complex dialog between the host and the pathogen

4.1. MICROSCOPY

The development of more sensitive microscopic tools to complement flow cytometric techniques has been essential for the determination of whether particular genes have a specific impact on the host and host cellular response. Recent research has shown that pathogens can interfere with normal host cell-mediated immune response by modifying the inflammatory MAP-Kinase-based cascade that normally leads to the production of cytokines and the elimination of the pathogen (Galan, 1999; Hobbie *et al.*, 1997). More and more examples show specific interactions of bacterial virulence factors (“effector” proteins) and host signalling determinants. This reinforces the need to investigate each step of bacterial pathogenesis from the viewpoint of both the bacteria and the host. Microscopic approaches for visualising infection have evolved considerably, and the increasing availability of antibodies specific to bacterial- or host-derived determinants has improved our understanding of natural infection processes. Light microscopy has been used for comparing the histopathology of mice infected with wild type *S. Typhimurium* or with a *S. Typhimurium* mutant strain not capable of repressing the production of host γ -IFN (Valdivia *et al.*, 2000). Conventional microscopy has also been used to demonstrate that mycobacterial surface proteins are released from bacterial phagosome in infected macrophages (Beatty and Russell, 2000). Recent advances in optical

imaging systems have now been successfully applied to answer important microbiological questions (see below), and involve cooled Charged-Couple-Device (CCD) cameras capable of generating colour image data sets containing more information than available from a 35 mm colour reversal film. CCD cameras allow improved spatial resolution, combined with geometric and photometric linearity (Cinelli, 1998, Fung and Theriot, 1998; Entwistle, 1998). Acquisition and data storage capabilities have also been improved; the replacement of film with digital technology has improved image quality by facilitating background subtraction and contrast enhancement on both 2D and 3D images. Dedicated software is now available to perform signal intensity comparison from microscopic images, but this does not yet provide a reliable tool for accurate quantification of gene expression from reporter fusions.

Microscopes now offer much more sensitive detection of weak signals than a few years ago. Confocal microscopes have been developed to only register light from the focal plane, removing the problem of light contamination from out of focus regions. This approach has allowed the co-localisation of bacteria with host specific markers. Examples include the proof that *S. Typhimurium* cells are found within phagocytic cells in liver sections (Richter-Dahlfors *et al.*, 1997), and are co-localised with the LAMP-1 vacuolar membrane protein of infected cultured cells (Beuzon *et al.*, 2000; Rathman *et al.*, 1997). Confocal microscopy has also been used to observe interference of *Salmonella* with normal host cell structure, such as the tight junctions in MDCK epithelial cells (Jepson *et al.*, 2000).

Multiphoton microscopy promises to revolutionise the field by overcoming the problem of photobleaching. The technique involves treating the specimen with a stream of pulsed infrared light, at a pulse frequency that allows one dye molecule to absorb two photons at once. This happens only at the focal plane. Combining the energy of the two long-photons brings the dye to its excitation state, generating fluorescence (Denk *et al.*, 1990). The two-photon approach allows photo-bleaching to be confined to the vicinity of the focal plane, and allows

sharp signal localisation with greatly improved image quality. However, microscopy is generally regarded as a qualitative rather than a quantitative technique.

4.2. FLOW CYTOMETRY

It is clear from this review and other studies that bacterial gene expression needs to be accurately measured *in vivo*, and flow cytometry is proving to be an ideal tool for quantification. The process of flow cytometry involves the carriage of sample cell suspensions in a fluidic system and passage through a laser beam. Forward- and side-scattered light is collected to give information about size and density of each cell. If the cells harbour any fluorescent molecules, the fluorescence intensity of each individual particle is amplified, collected and saved. Flow cytometric approaches are ideal for measuring expression in individual eukaryotic cells initially and remain a powerful tool to monitor a wide range of cellular functions including immune cell maturation or cytokine production. The use of flow cytometry has been developing in the microbiological field, and is becoming increasingly important for measuring population heterogeneity, cell cycle and membrane integrity etc (Davey and Kell, 1996; Nebe-von-Caron *et al.*, 2000). Flow cytometry has also been used in studies of pathogenesis, either to follow intracellular spread of pathogens, such as *Shigella flexneri* (Rathman *et al.*, 2000) or to detect bacterial gene expression in individual macrophages, as described for DFI (Valdivia and Falkow, 1997). FACS is ideal for the enrichment of cellular subpopulations. However, flow cytometric analysis and sorting of bacteria is complicated by the small size of bacterial cells, which is close to the current limit of detection (Davey and Kell, 1996; Shapiro, 1995). Nevertheless, analysing and sorting has been reported for many bacterial species (Hewitt *et al.*, 2000; Vives-Rego *et al.*, 2000). Several examples of the use of flow cytometry to monitor bacterial gene expression have been reported in pathogens including several Mycobacterial species (Kremer *et al.*, 1995, Valdivia and Falkow, 1996; Valdivia and Falkow, 1997; Valdivia and Ramakrishnan, 2000).

The first combination of *in situ* hybridisation with flow cytometry has recently been achieved (Chen *et al.*, 2000). RT-PCR was used to reverse transcribe mRNA and to amplify cDNA generated from *P. putida* grown in inducing conditions for expression of the gene of interest, a toluene dioxygenase (*todC1*). PCR products were labelled with fluorescence and used as probes for *in situ* hybridisation experiments inside *Pseudomonas* cells. Analysis in flow cytometry of hybridised cells allowed visualisation of *todC1* induction as well as indirect estimation of its expression levels in each bacterial cell. Flow cytometry is likely to be the key technique for measuring gene expression in individual bacterial cells.

4.3. GLOBAL EXPRESSION PROFILING

The availability of the Human UniGene database (<http://www.ncbi.nlm.nih.gov/UniGene/>) and the recent completion of a number of bacterial pathogen genome sequences allow us to monitor mammalian and bacterial transcription at the genomic scale using DNA microarrays, and to produce a “gene expression profile” for a particular organism under certain environmental conditions (DeRisi *et al.*, 1996; Hughes *et al.*, 2000; Lockhart *et al.*, 1996; Schena *et al.*, 1995).

Mammalian gene microarrays have been used to study host-pathogen interactions from the host’s viewpoint, by identifying gene expression patterns induced by the presence of a pathogen (Manger and Relman, 2000). Several *in vitro* studies have explored the effects of infection by *L. monocytogenes* and *S. Typhimurium* on the human cell response (Cohen *et al.*, 2000; Eckmann *et al.*, 2000; Rosenberger *et al.*, 2000). These authors observed an up-regulation of certain genes including several cytokines, kinases, HLA-ClassI and transcriptional factors. Other groups have used a more complex approach to compare host response induced by pathogenic strains of bacteria carrying well-defined mutations (Manger and Relman, 2000).

We require improved technology for the *in vivo* study of infection with microarrays, both from the host’s and the pathogen’s point of view. Advances have been recently made in

applying the linear RNA amplification method to extract sufficient eukaryotic mRNA for microarray analyses from small amounts of mammalian tissue (Gonzalez *et al.*, 1999; Wang *et al.*, 2000). However, the use of microarrays for the study of bacterial infection at the level of gene expression is still in its infancy (Cummings and Relman, 2000; Hautefort and Hinton, 2000). The lack of polyadenylated mRNA complicates the isolation of sufficient, good quality bacterial mRNA from complex environments such as mammalian tissue. The successful application of a linear RNA amplification approach to bacterial mRNA on a genomic scale has yet to be reported.

Consequently, the alternative technology of “real-time” RT-PCR is likely to become the current method of choice for direct monitoring bacterial mRNA during infection. Bubert *et al* described the use of RT-PCR to monitor *Listeria* gene expression in cultivated mammalian cells (Bubert *et al.*, 1999). The combination of this approach with “real time” PCR (Wei *et al.*, 2001) should result in a sensitive and widely-applicable technique.

4.4. TISSUE CULTURE MODELS

Although it is ideal to work with genuine animal models of infection, these do not exist for many pathogens. Therefore, cultivated cell-lines still have an important role for understanding microbial pathogenesis. More human or animal cell-lines are becoming available, and are generating intriguing results (see Table 2). *S. Typhimurium* benefits from being studied both in cultivated mammalian cells and in the mouse typhoid animal model. Until now, *Salmonella* gene expression has only been studied in cultivated cell lines particularly in the epithelial cells, dendritic cells or macrophages that are encountered by the bacteria during actual host infection. Animal models have been used to study the trafficking of *Salmonella* in host tissue, and to identify new bacterial *ivi* genes. Research on specific cultivated cell lines has

proved invaluable for developing our understanding of *Salmonella* pathogenesis, as summarised below.

Host invasion by *S. Typhimurium* not only occurs in epithelial cells (Finlay *et al.*, 1989) but also in phagocytic cells which have been shown to be able to capture *Salmonella* in the gut lumen (Vazquez-Torres *et al.*, 1999). Cultivated epithelial cell-lines have been used to demonstrate that actin rearrangements in the host cell cytoskeleton were induced by *Salmonella*, resulting in characteristic membrane ruffling (Galan and Zhou, 2000; Ginocchio *et al.*, 1994). Actin rearrangement is caused by the *Salmonella* effector, SopE, which is an exchange factor for RhoGTPases (Hardt *et al.*, 1998). Epithelial cells have also been used to show that *Salmonella* participates actively in the restoration of the normal structure of the host cell cytoskeleton after its entry. This involves a second effector protein, SptP, that acts at the level of two RhoGTPases Dc42 and Rac1 (Fu and Galan, 1999). Recently, polarised intestinal epithelial cells have been used to observe the induction of the pro-inflammatory response by *S. Typhimurium* (Gewirtz *et al.*, 1999). The authors have shown that translocation of flagellin across the intestinal epithelial cells was responsible for this induction at the basolateral surface of the cell layer.

Following invasion, *Salmonella* encounters various other immune cell types in the murine model. Macrophages have long been regarded as the main target encountered by *S. Typhimurium*. Recently, Niedergang and collaborators have demonstrated that the uptake of *Salmonella* by dendritic cells does not depend on the same virulence factors required for uptake by macrophages (Niedergang *et al.*, 2000). Furthermore, we now know that *S. Typhimurium* is capable of inducing the maturation of bone marrow-derived dendritic cells, causing reduction in their capability to present antigens from subsequently encountered pathogens (Svensson *et al.*, 2000). Similar observations were also made in *Mycobacterium bovis* BCG-infected dendritic cells (Tsuji *et al.*, 2000).

Most pathogens will encounter macrophage-mediated phagocytosis at some stage of the infection process. Cultivated macrophages have been used to study several other pathogens, including *Listeria monocytogenes*, and led to the surprising discovery that OppA, encoded by a gene belonging to the oligopeptide permease operon, was important for intra-cellular survival (Borezee *et al.*, 2000). Cultured cell models allow us to investigate how the pathogen can escape the normal host immune response. One strategy relies on interference with cytokine production by factors such as *Salmonella* LPS lipid A. PhoPQ-regulation of the structural modification of Lipid A is directly responsible for inducing expression of the E-selectin adhesion molecule in endothelial cells, and TNF- α in adherent monocytes (Guo *et al.*, 1997). In macrophages, SifA has recently been shown to be the bacterial determinant responsible for maintaining membrane integrity of the *Salmonella*-containing vacuole (Beuzon *et al.*, 2000).

However, the use of cultured cells does present limitations for the analysis of bacterial and host responses: i) most phagocytic and invasion assays require recently isolated primary cells, such as macrophages or bone-marrow derived dendritic cells, to obtain biologically-relevant data. It is clear that differentiated macrophages rapidly lose important functions associated with host immune response during passage, preventing the acquisition of accurate and reliable data. ii) Results can differ significantly between individual cell lines, even though they are all derived from the same cell type. A good example of this is the comparison of the importance of vacuole acidification on *S. Typhimurium* survival in different macrophage cell lines. Acidification is unnecessary for pathogen survival in RAW264.7 macrophages, but is crucial in J774 macrophages (Rathman *et al.*, 1996; Steele-Mortimer *et al.*, 2000). This highlights the danger of extrapolating from observations made on a simplified model to understand what happens during infection. iii) Pure cultures of mammalian cells are generally grown in stabilised media. This situation is completely different to host tissue, where different cell types co-exist and share cross-talk. In summary, cultivated cell lines are convenient for

preliminary studies but the results obtained must be confirmed in more complex systems such as animal models, when possible.

4.5. ANIMAL MODELS

The limitations of research based on cell lines described in section 4.4 have fuelled interest in relevant animal models. We know that the host represents a complex and dynamic environment, which is modified during the infection process, presenting a variety of stimuli to which the pathogen must respond if it is to be successful. Techniques like IVET, STM, Subtractive Hybridisation or Differential Display have been extensively used with animal models to identify *ivi* genes and the use of these models to focus on the host/pathogen interaction is developing rapidly.

Techniques previously used for *ivi* gene identification are now commonly combined with other approaches to study the host/pathogen dialogue. Differential Display has been used in combination with *in situ* hybridisation to characterise differential expression of nervous tissue genes upon *Herpes simplex* virus infection (Wilkinson *et al.*, 2000). Differential Display was used to identify cDNA derived from nervous tissue-induced genes, and was subsequently used as probe for *in situ* hybridisation experiments on nervous tissue sections. This approach allowed co-localisation of transcript expression with specific cell-types of the host tissue, and should prove applicable to the identification of pathogen genes exhibiting cell-type specific expression patterns in the infected host.

The role of identified bacterial virulence determinants in animal infection has often been determined by studying the virulence attenuation of particular mutants, as reported for many *S. Typhimurium* studies (Deiwick *et al.*, 1998; Shea *et al.*, 1999). This strategy identifies the stage of pathogenesis at which a particular gene is required, and has been used effectively in combination with competitive index (Unsworth *et al.*, 2000). Recently, inactivation of a

DFI-identified gene of *S. Typhimurium*, *mig14*, was shown to have no effect on bacterial replication in host tissue early after infection, but to cause rapid clearance from spleen and liver at a later stage (Valdivia *et al.*, 2000). This suggests that *mig14* might be involved in survival of the bacteria in deep tissue.

However, the use of bacterial mutant studies to obtain indirect evidence is not sufficient to establish the role of specific genes in infection. It is crucial to determine where microbial virulence genes are expressed during infection, whether virulence genes exhibit organ- or cell type-specific expression patterns and if their expression varies from one stage of infection to another. This approach requires an optimised reporter system to visualise bacterial gene expression directly in host tissue. The system we have developed is being used to assess the transcriptional response of *Salmonella* SPI1, SPI2 and other virulence genes during mouse infection. This IVIF approach (*In Vivo* Induction of Fluorescence) (Hautefort and Hinton, 2000) uses the bright and stable GFP⁺ (section 3.2) as a transcriptional reporter gene that is fused to various virulence gene promoters and integrated into the chromosome at single copy. In both cultivated cells and in mouse infected liver and spleen, GFP fluorescence can be detected and quantified by fluorescent microscopic and flow cytometric techniques respectively. This GFP-based reporter system allows the study of single bacteria directly isolated from an infected host. Furthermore, IVIF facilitates the co-localisation of bacterial gene expression with specific host immune markers. Figure 1 shows expression of *ssaG::gfp* within CD-18 labelled phagocytic cells in a section of infected mouse spleen.

Although animal models present many advantages over cultivated cell systems, a major limitation exists for human pathogens: most animal models only partially reproduce the symptoms observed in human infections. Indeed, in many instances, the animal model used does not closely resemble the condition found in human host and erroneous conclusions exist in the literature resulting from extrapolation from animal model to human (Smith, 1998).

Salmonella has been extensively studied in mouse, although this model does not resemble human gastroenteritis. Very little information is available on the interaction of *S. Typhimurium* with the epithelium of the gastrointestinal (GI) tract in humans, as almost all available data come from animal studies and cell culture models (Wallis and Galyov, 2000); current knowledge of the pathogenesis of *Salmonella* in humans is limited to observations from clinical or typhoid fever and experimental infection of volunteers (Hornick *et al.*, 1970a; Hornick *et al.*, 1970b; Santos *et al.*, 2001; Tsolis *et al.*, 1999). Access to healthy human tissue samples is limited, and development of improved models must be a priority. We should remember that animal models, which mimic natural pathogen reservoirs (chicken, pigs, calves), are very useful for monitoring bacterial behaviour prior to human infection (Bispham *et al.*, 2001; Tsolis *et al.*, 1999). New animal models of increased relevance are being developed for pathogens initially studied in a different model, such as the use of chicken jejunal loops to study intestinal epithelial invasion by *S. enterica* (Aabo *et al.*, 2000). Intravenous infection of calves and mice by *S. Dublin* has recently allowed comparison of the behaviour of the same SPI2 mutants in different animal hosts (Bispham *et al.*, 2001). In both animal models, the *sseD* and *ssaT* mutants tested were attenuated, although still capable of invading bovine ileal loops.

The knock-out mouse is becoming the animal model of choice for dissecting pathogen interaction with the host immune system. The inactivation of certain modulators of the immune response, such as cytokines in knockout mice, allows their effects on bacterial pathogenesis to be established. Macrophage apoptosis has been described as a common mechanism for evading the immune system and facilitating intracellular survival by *Salmonella* (Monack *et al.*, 1996). Induction of this programmed cell-death by *Salmonella* requires a mechanism that is under investigation. Macrophages have been purified from Caspase-1 knock-out mice, and used for cytotoxicity and binding assays with *Salmonella*, in comparison with macrophages derived from a wild-type mouse. This study showed that the *Salmonella* SipB effector protein induces

macrophage apoptosis by binding to the pro-apoptotic enzyme caspase-1 (Hersh *et al.*, 1999; Monack *et al.*, 2000). Knock-out mouse studies have been used to identify two other factors involved in the inhibition of intracellular proliferation by *Salmonella*. Phagocyte oxidase (Phox) and inducible nitric oxide synthase (iNOS) are two enzymes involved in the synthesis of reactive bactericidal oxidants. Phox and iNOS knock-out mouse experiments confirmed a role of these enzymes in microbial proliferation and host survival of *S. Typhimurium* (Mastroeni *et al.*, 2000). Subsequently, the authors showed that SPI2 effector proteins, such as SseB, are involved in targetting iNOS away from the *Salmonella*-containing vacuole (Vazquez-Torres *et al.*, 2000). The host response cascade is also being dissected by introducing the use of appropriate antibodies into the animal model (Mastroeni *et al.*, 1998).

5. Conclusion

Studies at the level of molecular pathogenesis have considerably accelerated the pace of understanding bacterial infection processes. A wide range of powerful techniques is now available in this field of cellular microbiology, and interest is now extending to pathogens for which very few genetic tools have been available.

IVET, DFI and STM approaches have revolutionised studies of bacterial infection, allowing indirect selection of genes whose expression occurs during infection. Subtractive Hybridisation, Differential Display and alternative techniques like SAGE or SCOTS are appealing because they facilitate direct monitoring of mRNA induced during infection. Proteomics allows the correlation of changes in levels of transcription of *ivi* genes with direct analysis of IVI proteins. Immunological-based techniques allow us to look directly at human infections, and to identify IVI proteins that are definitely involved in human infectious disease.

The rapid development of relevant tissue culture cell lines, new animal models, highly-sensitive microscopic and flow cytometric equipment combined with the increased

availability of specific antibodies, fluorescent probes and reporter genes has had considerable impact on our ability to understand infection processes *in situ*. Analysis of gene expression in individual micro-organisms is now possible with IVIF for *S. Typhimurium*, and should be applicable to a wide range of pathogens.

The revolutionary development of DNA microarray technology provides a complementary approach for monitoring global levels of gene expression during infection. Microbial infection studies are moving towards a new post-genomic era that will certainly provide new insights into host/pathogen interactions.

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Figure 1: Spleen section of BALB/c mouse infected with *S. Typhimurium*.

Salmonella cells that carry a single copy *ssaG::gfp* transcriptional fusion in the chromosomal *put* locus are detected with an anti-LPS antibody (red fluorescence). An anti-CD18 monoclonal antibody (blue fluorescence) identifies all phagocytic cells. Green fluorescence reflects *ssaG::gfp* expression within phagocytic cells.

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Organism	Technique used	Model	Reference
<i>Actinobacillus pleuropneumoniae</i>	STM	Porcine infection	(Fuller <i>et al.</i> , 2000b)
<i>A. pleuropneumoniae</i>	IVET	Porcine infection	(Fuller <i>et al.</i> , 1999)
<i>Aspergillus fumigatus</i>	STM	Invasive pulmonary infection	(Brown <i>et al.</i> , 2000)
<i>Bartonella henselae</i>	DFI	HEp-2 cell invasion	(Lee and Falkow, 1998)
<i>Bordetella bronchiseptica</i>	Differential Display	<i>In vitro</i> induced Bvg+ infectious phase	(Yuk <i>et al.</i> , 1998)
<i>Brucella suis</i>	STM	Human macrophage	(Foulongne <i>et al.</i> , 2000)
<i>Clostridium perfringens</i>	Differential Display	Wild-type v/s virR mutant	(Banu <i>et al.</i> , 2000)
<i>Escherichia coli</i>	Differential Display	Heat-shocked cultures	(Gill <i>et al.</i> , 1999)
<i>E. coli K1</i>	STM	Human brain vascular endothelial cells	(Badger <i>et al.</i> , 2000)
		Gastrointestinal tract of infant rats	(Martindale <i>et al.</i> , 2000)
<i>Histoplasma capsulatum</i>	IVET	Mouse & Cultivated cells	(Retallack <i>et al.</i> , 2000)
<i>Legionella pneumophila</i>	Differential Display	Macrophage infection	(Abu Kwaik and Pederson, 1996)
<i>Listeria monocytogenes</i>	IVET	Mouse	(Gahan and Hill, 2000)
			(Dubail <i>et al.</i> , 2000)
<i>M. tuberculosis</i>	Differential Display	Murine Macrophage Infection	(Ragno <i>et al.</i> , 1998)
<i>M. marinum</i>	DFI	Macrophage infection	(Barker <i>et al.</i> , 1998)
			(Ramakrishnan <i>et al.</i> , 2000)
<i>M. tuberculosis</i>	DFI	Macrophage infection	(Triccas <i>et al.</i> , 1999)
<i>M. tuberculosis</i>	SCOTS	Human macrophage	(Graham and Clark-Curtiss, 1999)
<i>M. tuberculosis</i>	Subtractive Hybridisation	Macrophage infection	(Plum and Clark-Curtiss, 1994)

<i>Pasteurella multocida</i>	STM	Septicaemia in mouse	(Fuller <i>et al.</i> , 2000a)	<i>Staphylococcus aureus</i>	Subtractive Hybridisation	Identification of DNA sequences specific to virulent clinical isolates	(El-Adhami, 1999)
<i>Proteus mirabilis</i>	STM	CBA mouse	(Zhao <i>et al.</i> , 1999)				
<i>Pseudomonas aeruginosa</i>	IVET	BALB/c mouse	(Handfield <i>et al.</i> , 2000)	<i>Streptococcus agalactiae</i>	STM	Neonatal rat sepsis	(Jones <i>et al.</i> , 2000)
		Respiratory mucus derived from Cystic Fibrosis patients	(Wang <i>et al.</i> , 1996a)	<i>Streptococcus gordonii</i>	IVET	Rabbit endocarditis model	(Kilic <i>et al.</i> , 1999)
		Rat lung infection	(Wang <i>et al.</i> , 1996b)	<i>S. gordonii</i>	IVET	Acidic pH	(Vriesema <i>et al.</i> , 2000)
			(Handfield <i>et al.</i> , 2000)	<i>S. gordonii</i>	Differential Display	Saliva v/s brain Heart Infusion	(Du and Kolenbrander, 2000)
		Murine burn wound infection	(Ha and Jin, 1999)	<i>Streptococcus pneumoniae</i>	STM	BALB/c mouse	(Polissi <i>et al.</i> , 1998)
<i>Pseudomonas fluorescens</i>	IVET	Rhizosphere	(Rainey, 1999)	<i>Vibrio cholerae</i>	STM	Suckling mouse	(Chiang and Mekalanos, 1998)
<i>Salmonella enterica</i> sv. Typhimurium	DFI	Acid shock	(Valdivia and Falkow, 1996)	<i>Yersinia enterocolitica</i>	STM	BALB/c mouse	(Darwin and Miller, 1999)
		Macrophage infection	(Valdivia and Falkow, 1997)				
<i>Salmonella typhi</i>	IVET	Henlé cells	(Staendner <i>et al.</i> , 1995)				
<i>S. Typhimurium</i>	STM	BALB/c mouse	(Hensel <i>et al.</i> , 1995)				
<i>S. Typhimurium</i>	IVET	Peyer's patches	(Stanley <i>et al.</i> , 2000)				
<i>S. Typhimurium</i>	IVET	Murine hepatocyte	(Janakiraman and Schlauch, 2000)				
<i>S. Typhimurium</i>	SCOTS	Macrophage infection	(Morrow <i>et al.</i> , 1999)				
<i>S. Typhimurium</i>	Proteomics	Macrophage infection	(Deiwick and Hensel, 1999)				
<i>Small intestine and colonic epithelial cells</i>	Differential Display	Association with a gut microflora	(Ogawa <i>et al.</i> , 2000)				
<i>Staphylococcus aureus</i>	STM	Mouse abscess	(Coulter <i>et al.</i> , 1998)				
		Bacteremia in mouse	(Mei <i>et al.</i> , 1997)				
		Infected wounds in mouse & endocarditis in Rabbit	(Schwan <i>et al.</i> , 1998)				
<i>S. aureus</i>	IVET	Milk	(Lammers <i>et al.</i> , 2000)				
<i>S. aureus</i>	IVET	Murine renal abscess	(Lowe <i>et al.</i> , 1998)				

Table 2: Bacterial determinants involved in Host/Pathogen interaction

Organism	Host/pathogen interaction	Host & pathogen factors involved	Reference
<i>Campylobacter jejuni</i>	Release of IL-8 by human intestinal epithelial cells	Adherence and/or invasion & Cytolethal Distending Toxin (CDT)	(Hickey <i>et al.</i> , 2000)
Group A <i>Streptococcus</i>	Adhesion to human epithelial cells	Scl	(Lukomski <i>et al.</i> , 2000)
<i>Haemophilus ducreyi</i>	Arrest of infected cells in mitosis only after internalisation of HdCDT	Hd Cytolethal Distending Toxin	(Cortes-Bratti <i>et al.</i> , 2000)
HIV type 1	Chemokine coreceptor CCR-5 phenotypic knockout leads to infection resistance to HIV-1	Unknown	(Yang <i>et al.</i> , 1997)
<i>Klebsiella pneumoniae</i>	Adhesion to & invasion of epithelial cells	Capsule	(Sahly <i>et al.</i> , 2000)
<i>Legionella pneumophila</i>	Phagosome-related stress such as Thymine limitation	Rep Helicase	(Harb and Abu Kwaik, 2000)
<i>Listeria monocytogenes</i>	Intra-macrophage survival and bacterial growth in infected murine organs	OppA	(Borezee <i>et al.</i> , 2000)
<i>Mycobacterium bovis</i> BCG	Dendritic cell maturation	Peptidoglycan, arabinogalactan and mycolic acids	(Tsuji <i>et al.</i> , 2000)
<i>Salmonella typhi</i>	IL6 production by epithelial cells	Unknown	(Weinstein <i>et al.</i> , 1997)
<i>Salmonella</i> Typhimurium	Dendritic cell maturation	LPS	(Svensson <i>et al.</i> , 2000)
<i>S. Typhimurium</i>	Actin cytoskeleton rearrangement via Cdc42 and Rac1 RhoGTPases in rat Ref52 cells	SptP	(Fu and Galan, 1999)
<i>S. Typhimurium</i>	Membrane ruffling via actin rearrangement	SopE	(Hardt <i>et al.</i> , 1998)
		SopE2	(Bakshi <i>et al.</i> , 2000)
<i>S. Typhimurium</i>	LPS-mediated expression of E-selectin and TNF α by endothelial cells and adherent monocytes	LPS-lipidA	(Guo <i>et al.</i> , 1997)

endothelial cells and adherent monocytes

respectively

<i>S. Typhimurium</i>	Invasion repression by bile	BarA/SirA	(Prouty and Gunn, 2000)	<i>Uropathogenic E. coli</i>	CD55 & CD66e clustering around the bacteria and	Dr-II adhesin	(Guignot <i>et al.</i> , 2000)
<i>S. Typhimurium</i>	Fusion of bacterial and host cell outer membranes	SipB	(Hayward <i>et al.</i> , 2000)		apical actin rearrangement in polarised epithelial		
<i>S. Typhimurium</i>	Rapid and delayed induction of apoptosis in macrophages	SipB, InvA and OmpR, SsrB, SpiB respectively	(van Der Velden <i>et al.</i> , 2000)		Caco-2/TC7 cells		
<i>S. Typhimurium</i>	Apoptosis of Macrophages	Caspase 1 & SipB	(Hersh <i>et al.</i> , 1999)	<i>Vibrio vulnificus</i>	Adherence to HEp-2 epithelial cells and virulence in	PilD	(Paranjpye <i>et al.</i> , 1998)
<i>S. Typhimurium</i>	Aggregation of host endosomal compartments into <i>Igp</i> -tubules	SpiC, SseFG, SsaJ,L,M,V,P, SpvR, SifA, <i>carA</i> associated auxotrophy	(Guy <i>et al.</i> , 2000)		iron-overloaded mice		
<i>S. Typhimurium</i>	Induction of inducible Nitric Oxide Synthase (iNOS) expression	SipB,C,D & SopE2	(Cherayil <i>et al.</i> , 2000)				
<i>S. Typhimurium</i>	Prevention of co-localisation of iNOS and <i>Salmonella</i> in phagocytic cells in mouse organs	SPI-2 proteins like SseB	(Vazquez-Torres <i>et al.</i> , 2000)				
<i>S. Typhimurium</i>	Bacterial interference with normal cytokine production such as γ IFN	Mig14	(Valdivia <i>et al.</i> , 2000)				
<i>S. Typhimurium</i>	Induction of TNF α expression in human promonocytic cells	FliC	(Ciacci-Woolwine <i>et al.</i> , 1998)				
<i>S. Typhimurium</i>	HEp-2 epithelial cells	CsrA	(Altier <i>et al.</i> , 2000)				
<i>S. Typhimurium</i>	Mouse intestinal colonisation	LPS	(Licht <i>et al.</i> , 1996)				
<i>S. Typhimurium</i> & Dublin	Apoptosis-independant Macrophage lysis	Caspase 1 & SipB	(Watson <i>et al.</i> , 2000)				
<i>Shigella flexneri</i>	IL-1 β production by macrophages	IpaB invasin	(Thirumalai <i>et al.</i> , 1997)				
<i>Ureaplasma urealyticum</i>	Activation of NF- κ B and induction of iNOS in macrophages	Unknown	(Li <i>et al.</i> , 2000)				

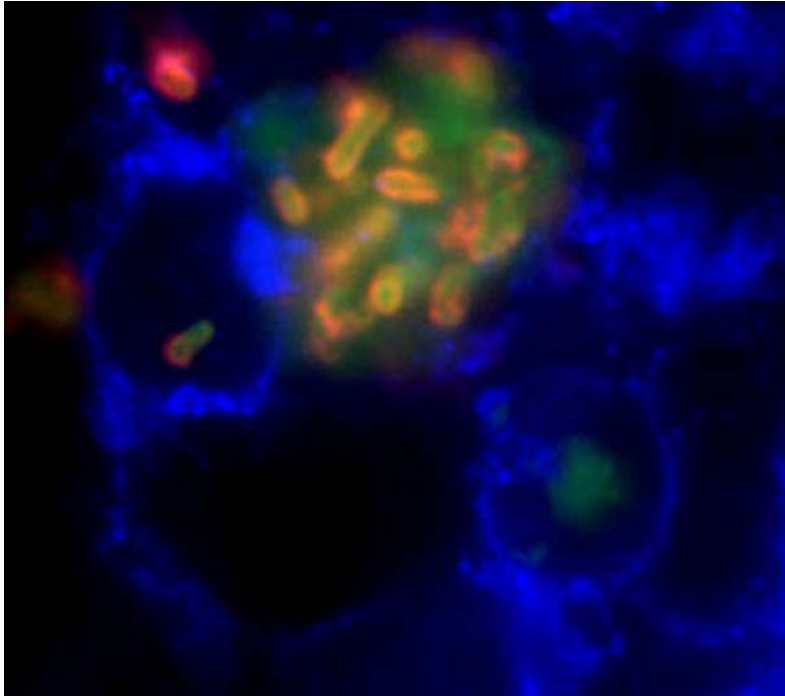


Figure 4.1: Spleen section of BALB/c mouse infected with *S. Typhimurium*.

Salmonella cells that carry a single copy *ssaG::gfp* transcriptional fusion in the chromosomal *put* locus are detected with an anti-LPS antibody (red fluorescence). An anti-CD18 monoclonal antibody (blue fluorescence) identifies all phagocytic cells. Green fluorescence reflects *ssaG::gfp* expression within phagocytic cells.