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THE ROYAL SOCIETY

Identification and analysis of bacterial virulence genes *in vivo*

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We dedicate this paper to our friend and colleague Geoffrey Banks (1939–1999)

Signature-tagged mutagenesis is a mutation-based screening method for the identification of virulence genes of microbial pathogens. Genes isolated by this approach fall into three classes: those with known biochemical function, those of suspected function and some whose functions cannot be predicted from database searches. A variety of *in vitro* and *in vivo* methods are available to elucidate the function of genes of the second and third classes. We describe the use of some of these approaches to study the function of the *Salmonella* pathogenicity island 2 type III secretion system of *Salmonella typhimurium*. This virulence determinant is required for intracellular survival. Secretion by this system is induced by an acidic pH, and its function may be to alter trafficking of the *Salmonella*-containing vacuole. Use of a temperature-sensitive non-replicating plasmid and competitive index tests with other genes show that *in vivo* phenotypes do not always correspond to those predicted from *in vitro* studies.

Keywords: signature-tagged mutagenesis; SPI-2; type III secretion; competitive index

1. INTRODUCTION

The study of bacterial mutants *in vivo* can provide important insights into the diverse mechanisms of virulence employed by pathogenic organisms. Signature-tagged mutagenesis (STM) is one such approach, enabling the identification of virulence genes from a variety of pathogens. The original STM screen identified the *Salmonella* pathogenicity island 2 (SPI-2) type III secretion system (TTSS). The attenuated virulence of SPI-2 mutants has been the starting point for further analysis of the secretion system by our group and others. The function of SPI-2 was not obvious from its sequence, and various *in vitro* assays have been carried out to investigate the TTSS in greater detail. In this paper we discuss the results of those experiments and others that have addressed the role of SPI-2 *in vivo*.

2. SIGNATURE-TAGGED MUTAGENESIS

STM was developed as an *in vivo* genetic screen for the identification of microbial virulence genes. The intention was to allow a relatively rapid unbiased search for virulence genes using an animal host to select against strains carrying mutations in genes affecting virulence, among a mixed population of mutants. Searches have been carried out by screening individual bacterial mutants in an appropriate host (Miller *et al.* 1989; Bowe *et al.* 1998), but this is a relatively labour intensive and expensive approach if large numbers of strains are to be screened. By tagging each mutant with a different DNA 'signature', STM allows large numbers of different strains to be screened at the same time in the same animal host.

STM is a comparative hybridization technique that employs as mutagens a collection of transposons or other recombinogenic transforming DNA molecules, each modified by the incorporation of a different DNA sequence tag. The tags were originally designed as short DNA segments containing a 40 bp variable central region flanked by invariant 'arms' that facilitate the co-amplification and labelling of the central portions by polymerase chain reaction (PCR). When the tagged mutagens integrate into the genome of an organism, each individual mutant can in theory be distinguished from every other mutant based on the different tags carried by the strains.

In STM, mutagenized strains are stored individually in arrays (usually in the wells of microtitre dishes), and colony or dot blots are made from these arrays, or from plasmids carrying the tags or from the tags themselves. Pools of mutants are then inoculated into an appropriate animal host, and PCR is used to prepare labelled probes representing the tags present in the inoculum (input) and recovered from the host (output). Hybridization of the tags from the input and output pools to the colony or dot blots permits the identification of mutants that fail to grow *in vivo*, because the tags carried by these mutants will not be present in the output pools. These strains can then be recovered from the original arrays and the nucleotide sequence of DNA flanking the mutation site can be determined (figure 1).

The STM approach has a number of inherent limitations. First, it relies on the ability of the pathogen in question to replicate *in vivo* as a mixed population, and can

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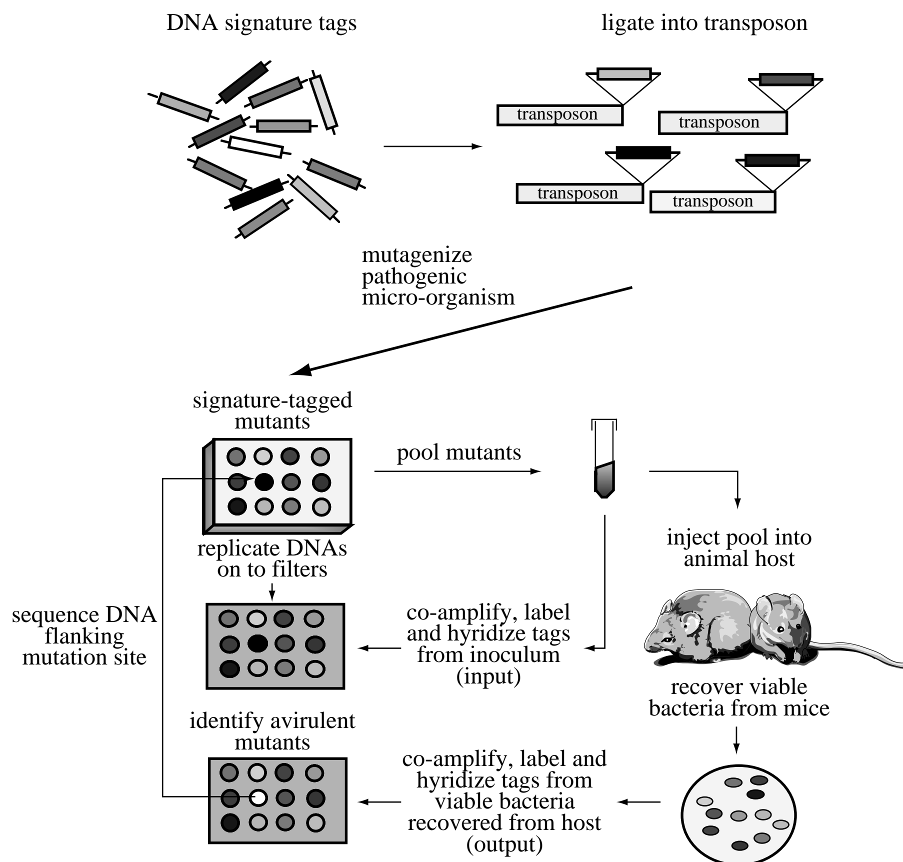

Figure 1. Principles of STM. A transposon is modified by incorporation of DNA signature tags. A collection of different insertional mutants of a bacterial pathogen, each carrying a different tag, is assembled in a microtitre dish. The mutants are pooled and used as the inoculum for an appropriate animal model of infection. After a period of time in which virulent bacterial strains have multiplied, bacteria are recovered from the host. Signature tags representing strains in the inoculum and strains recovered from the animal are separately amplified using primers that anneal to invariant sequences flanking the tags, then labelled and used to probe membranes carrying DNA from the mutants in the microtitre dish. An avirulent mutant is identified by its failure to yield a signal on the membrane hybridized with the tags recovered from the animal (Tang & Holden 1999).

only be expected to identify virulence genes whose mutant phenotypes cannot be transcomplemented by other virulent strains present in the same inoculum. The types of virulence genes that could fail to be recovered as a result of mixed infection include those which encode secreted toxins, and factors which interfere with a systemic immune response (by altering cytokine production, for example). Second, STM usually involves an animal model of a human disease, and these models cannot be expected to faithfully reproduce all of the features of the bacterial–human interaction. Third, as with any genetic screen involving single mutations, any gene whose loss can be compensated for by another (genetic redundancy) is unlikely to be identified. Fourth, it is self evident that mutant strains generated by insertional mutagenesis have to be viable in order to inoculate them into an animal host, so STM (along with any insertional mutagenesis system) cannot provide any information about genes that are essential for life (with the proviso that insertions into 3′-ends of genes may result in truncated proteins which lose part but not all of an essential function).

Therefore, even if the mutant bank to be screened is large, and the genome of the pathogen is saturated with mutations, not all virulence genes of interest are likely to be recovered using STM; however, the hope was that a sufficient number of interesting genes would be identified to provide the basis for long-term studies into their functions.

The stimulus for developing STM came from work in our laboratory on *Aspergillus fumigatus*, and in particular the demonstration that two fungal products thought (on the basis of circumstantial evidence) to be important in pathogenicity, were in fact dispensable for this fungus to cause lethal pulmonary infections in mice (Smith *et al.* 1994). However, it was obvious at the time of its development that STM of *Aspergillus* would be a formidable task, not least because of the lack of an efficient method for insertional mutagenesis, the large number of mutants required for a representative mutant bank, and other technical difficulties (Brown & Holden 1998). Instead the *Salmonella typhimurium*–mouse interaction was chosen as a model system in which to assess the feasibility of the approach. This was for three reasons. First, excellent and straightforward molecular genetic methods were available for insertional mutagenesis using modified transposons (de Lorenzo & Timmis 1994). Second, the mouse model of systemic infection had been used extensively by many groups, is considered a good model of human typhoid fever and was known to support growth of a mixed population of different strains simultaneously (Mahan *et al.*

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1993). Third, many virulence genes had already been identified in *S. typhimurium* (Groisman & Ochman 1994), and the successful re-isolation of these would help to validate the method.

In the original method, tags were incorporated into a mini Tn*5* transposon and their suitability was checked prior to use by hybridization of amplified, labelled tags to DNA colony blots from the *S. typhimurium* mutants used to generate the probes. Mutants whose tags failed to yield clear signals on autoradiograms were discarded, and those that gave good signals were reassembled into new pools for screening in animals (Hensel *et al.* 1995). Pools of 96 strains were inoculated via the intraperitoneal route into mice. Viable bacteria were recovered from the spleens of animals three days post-inoculation, and the proportions of different mutants assessed by hybridization analysis of tags. Approximately 4% of the mutants tested were provisionally identified as attenuated in virulence. DNA regions flanking the transposon insertion sites of attenuated mutants were then recovered for sequencing either by restriction digestion and plasmid cloning, or by inverse PCR. The insertion points of 16 of the attenuated strains mapped to a 40 kb pathogenicity island at 30.7 centisomes on the chromosome (Hensel *et al.* 1995; Shea *et al.* 1996). The locus was named SPI-2 to distinguish it from the well-studied *Salmonella* pathogenicity island 1 (SPI-1) at 63 centisomes. It should be noted that SPI-2 genes were independently identified by a genome comparison approach (Ochman *et al.* 1996) and by differential fluorescence induction (Valdivia & Falkow 1997).

3. VARIATIONS AND IMPROVEMENTS

The STM method was subsequently modified to avoid the pre-screening process (Mei *et al.* 1997). In this version, a series of tagged Tn*917* transposons were selected prior to mutagenesis of *Staphylococcus aureus* on the basis of efficient tag amplification and labelling, and lack of cross-hybridization to other tags. These modified transposons were then used separately to generate a large number of *S. aureus* mutants that were arrayed according to the tags they carry. As the same tags can be used to generate an infinite number of mutants, this obviates the need to pre-screen mutant strains for the suitability of tags. A second advantage is that since the identity of the tag in each mutant is known, hybridization analysis can be done using plasmid or tag DNA dot blots rather than colony blots. This increases the sensitivity of the assay and allows the use of non-radioactive detection methods (Mei *et al.* 1997). In a further modification of the technique, Cormack *et al.* (1999) first introduced each of 96 tags at a disrupted *URA3* locus of *Candida glabrata*. Then for each tagged strain, many different mutants were created by insertional mutagenesis, using a plasmid that simultaneously complemented the *URA3* mutation. Although in most cases the tags are not physically closely linked to the mutations, the DNA flanking the mutations could be cloned easily by plasmid rescue in *Escherichia coli*. STM or variations of it has now been applied successfully in several microbial pathogens, including *S. typhimurium* (Hensel *et al.* 1995), *S. aureus* (Mei *et al.* 1997; Coulter *et al.* 1998; Schwan *et al.* 1998), *Streptococcus pneumoniae* (Polissi *et al.* 1998), *Vibrio cholerae* (Chiang & Mekalanos 1998), *Proteus mirabilis* (Zhao *et al.* 1999), *Yersinia enterocolitica* (Darwin & Miller 1999), *Legionella pneumophila* (Edelstein *et al.* 1999) and *C. glabrata* (Cormack *et al.* 1999).

For successful application of STM, it is essential to consider a number of parameters in order to obtain reproducible hybridization patterns from different animals inoculated with the same pool of mutants. As the number of different mutant strains in a pool is increased, so is the probability that fully virulent mutants will not be recovered in sufficient numbers to yield hybridization signals, and this could result in false identification of attenuated mutants. When *S. typhimurium* was inoculated into the peritoneal cavities of mice, pools of 96 different mutants gave reproducible hybridization signals after three days of infection, whereas pools of 192 did not (Hensel *et al.* 1995). With *V. cholerae* in a colonization model in mice, it was necessary to reduce the complexity of the orally inoculated pool to 48 strains to give reproducible results (Chiang & Mekalanos 1998).

In some cases, it may be difficult to achieve reproducible output signals even if the inoculum dose is varied. At low doses, there may not be enough cells of any one virulent mutant to initiate a successful infection, but at high doses a failure to recover the same mutants from different animals probably reflects a bottleneck in the infection process that selects individual cells stochastically, and these then grow out as the infection proceeds. Attempting to solve this problem by further increasing the size of inoculum may overwhelm the animal's immune defences, resulting in rapid death of the host or the growth of mutant strains that would otherwise be attenuated.

The route of administration of the bacterial inoculum obviously plays a major role in determining the numbers of bacterial strains that reach the target organ(s) and tissues, and therefore the reproducibility of tag hybridization signals. For example, when 10^5 *S. typhimurium* cells representing a pool of 96 mutants were inoculated by the intraperitoneal route, reproducible hybridization signals were obtained for the vast majority of strains recovered from the spleens of infected animals. When the same inoculum was given orally, however, only a small percentage of mutants were subsequently found in the spleens, and the identity of these varied from animal to animal (D. W. Holden, unpublished data).

Another important aspect of the STM screening process is the time-point at which bacteria are recovered to prepare tags for hybridization analysis. If the incubation period is short, virulent cells may have had insufficient time to outgrow the attenuated strains to a degree that is reflected in a clear difference in hybridization signal intensity of tags on the blots. On the other hand, if the period is too long, then there may be a risk that some virulent strains may simply outgrow other virulent strains stochastically.

The parameters described above are obviously interrelated and must be optimized empirically for each pathogen–host interaction in order to obtain reproducible hybridization patterns using tags recovered from at least two animals infected with the same pool of mutants.

In the STM study of *S. aureus* by Mei *et al.* (1997), 1248 Tn*917* mutants were tested in a murine model of bacteraemia. The majority of loci from 50 mutants that were

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identified as attenuated were predicted by sequence similarity to be involved in cell surface metabolism (e.g. peptidoglycan cross-linking and transport functions), nutrient biosynthesis and cellular repair processes, but most of the remainder had no known function. A slightly larger signature-tagged mutant bank was constructed using the same transposon and tested in models of bacteraemia, abscess and wound formation and endocarditis (Coulter *et al*. 1998). This enabled the identification of various genes affecting growth and virulence in specific disease states, as well as 18 that are important in at least three of the infection models. Many of these genes appear to be involved in the same kinds of processes as those identified in the earlier study; indeed, seven of the genes identified by Mei *et al*. (1997) were also found by Coulter *et al*. (1998).

It is curious that whereas many of the genes identified by STM in Gram-negative bacteria are clearly 'virulence determinants' in the classical sense of the term, many of the *S. aureus* genes identified by STM appear to have more fundamental roles in bacterial metabolism. Genes for known virulence determinants, such as toxins, extracellular matrix-binding proteins and their regulators, such as *agr* (Lowy 1998), have not yet been identified by these screens (Mei *et al*. 1997; Coulter *et al*. 1998; Lowe *et al*. 1998). In the case of STM, transposon mutagenesis is not fully random and may have favoured mutation of certain areas of the chromosome over others. Second, mutation of genes encoding toxins may result in transcomplementable phenotypes. Third, the screens carried out to date are probably not saturating with respect to mutations. Fourth, the failure to identify genes for extracellular matrix-binding proteins for example, may be explained by genetic redundancy for these proteins (Greene *et al*. 1995). Also, STM may not identify mutations causing small reductions in survival.

4. FURTHER ANALYSIS OF MUTANT STRAINS

When a mutant strain of interest has been identified, it is of course very important to obtain as much DNA sequence information around the site of the insertional mutation as is possible. With the rapid completion of many bacterial genome sequencing projects this task should become increasingly easy. It is essential to confirm that the tagged mutation is the cause of virulence attenuation, either by transduction of a transposon, recreation of the mutation by directed mutagenesis, outcrossing, and/or complementation with a functional allele, to show that the mutation and the virulence phenotype are linked. The DNA sequence might also indicate if the gene is part of an operon, and if so the phenotype could be due to a polar effect on a downstream gene. Directed mutagenesis of downstream genes is then necessary to establish which gene(s) are the cause of the attenuation.

Genes identified by an STM screen can be classified into three groups: first those whose products have a known biochemical function or whose function can be predicted on the basis of sequence similarities; second, genes with unknown specific function and relationship to virulence, but whose sequence gives some indication of functional class, such as an ATP-binding cassette transporter or a two-component regulatory system; and third, genes whose predicted amino-acid sequences give no clue to function. Several STM screens have identified genes of the first class. These are typically genes involved in biosynthesis of nutrients which are in limiting supply *in vivo*, such as amino acids and nucleotides (Mei *et al*. 1997). Knowledge that strains which lack the ability to synthesize a particular metabolite are attenuated in virulence is important because it can provide valuable information on the nutritional restrictions of different tissues *in vivo*. Furthermore, pathogens can sense the reduced availability of metals such as magnesium and iron in their extracellular environment and respond not only by synthesizing appropriate uptake systems but also by regulating other virulence factors (Garcia Vescovi *et al*. 1996; Dussurget & Smith 1998).

Table 1. *Analysis of virulence genes of unknown function*

virulence attenuation (LD_{50} and CI)
growth kinetics *in vivo*
starvation survival and growth rates in different media (auxotrophy?)
morphological abnormalities
physiological assays exploiting knowledge of pathogen–host interaction
- stress resistance (osmotic, pH, reactive N and O intermediates, antimicrobial peptides, etc.)
- resistance to complement and neutrophil killing
- adherence to extracellular matrix proteins or host cells
- invasion of host cells (if intracellular)

genetics: study of interactions with other genes by construction and analysis of double mutants *in vitro* and *in vivo*

To study the functions of genes which fall into the second and third classes, mutant strains can be subjected to a range of physiological assays, ranging from the general to the specific. Knowledge that the strain has a growth defect *in vivo* is a useful starting point. The types of assays which are most appropriate will vary from system to system, but some general questions that can be asked are listed in table 1. First the degree of virulence attenuation can be described in greater detail, using LD_{50} and the more sensitive and increasingly popular competitive index (CI) tests. *In vivo* studies can then be carried out with mutant strains (either as single or mixed infections with the wild-type parent strain) to describe the *in vivo* growth kinetics of the mutant and to identify the time and body site where the virulence defect is first apparent (Shea *et al*. 1999). The mutant strains can be tested for growth defects *in vitro*, in rich and minimal media, to establish if the phenotype is *in vivo* specific, and to identify an auxotrophy not apparent from the DNA sequence of the gene. Careful microscopic examination of the mutant strain may identify morphological abnormalities, such as a defect in motility or cell shape. A series of more specific physiological tests, using knowledge of the host–pathogen interaction, can also be carried out. Another approach that we have begun to use in our laboratory is the construction of double mutants and *in vivo* analysis of these to determine if the gene in question is functionally connected with another known virulence gene. An example of this approach is given below. In addition to these tests, molecular studies can be carried

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out using the genes themselves, such as construction of fusions with reporters to learn more about the timing and control of their expression, and the generation of antibodies to localize the protein within the bacterial cell and infected tissue, and possibly for immunoprecipitation experiments if the protein is suspected to interact with other bacterial or host proteins.

The remainder of this paper discusses the functions of a group of genes which fall into the second class, encoding the SPI-2 TTSS, with particular emphasis on the use of *in vivo* studies to help elucidate their function and relationship to other virulence factors required for systemic pathogenesis.

5. ORGANIZATION OF SPI-2 TTSS GENES

The discovery of the SPI-2 genes (Hensel *et al*. 1995) was surprising because it was already known that *S. typhimurium* has a TTSS (Inv/Spa) that is involved in epithelial cell entry. Indeed, *Salmonella enterica* appears to be unique among pathogenic bacteria in having two functionally distinct TTSS.

Comparison of the boundaries of SPI-2 with the corresponding region of the *E. coli* chromosome showed that SPI-2 is a 40 kb region of *Salmonella*-specific DNA inserted into a $tRNA^{val}$ gene, which is occupied in *E. coli* by 9 kb of *E. coli*-specific sequence (Hensel *et al*. 1997*a*). Hybridization analysis has demonstrated the presence of SPI-2 in the majority of serovars of *S. enterica*, but sequences hybridizing to the TTSS are absent from the ancestral species *Salmonella bongori* (Ochman & Groisman 1996; Hensel *et al*. 1997*a*). This is in contrast to the Inv/Spa TTSS of SPI-1, which is present in both species of *Salmonella* and was therefore probably acquired prior to SPI-2. A G+C content of 41.4%, which is considerably lower than the *Salmonella* genome average of 52%, suggests that SPI-2 was acquired by horizontal transfer from an unknown source. The pathogenicity island has a mosaic structure, with the TTSS encoded within the centisome 31 region. The centisome 30 region, which is not required for virulence, encodes genes involved in tetrathionate respiration (Hensel *et al*. 1999).

Four operons of SPI-2 appear to encode the secretion system; these have been designated 'regulatory', 'structural I', 'secretory' and 'structural II' (Hensel *et al*. 1997*b*; Cirillo *et al*. 1998). However, this nomenclature requires revision in the light of the recent finding that the *spiC* (also called *ssaB*) gene of the structural I operon encodes a secreted protein (Uchiya *et al*. 1999). Collectively, the products of 13 genes of the structural II operon are most similar to their homologues in the locus of enterocyte effacement of enteropathogenic *E. coli*, and are no more similar to the corresponding proteins from Inv/Spa than they are to those of the TTSS of the plant pathogen *Erwinia* (Elliot *et al*. 1998).

The principal proteins regulating SPI-2 gene expression form the two-component system SsrAB, and their genes comprise the regulatory operon. The products of *ssaL*, *ssaM* and *ssaP* of the structural II operon do not have significant similarity to products of other TTSS, and might be important for the specific function of the SPI-2 TTSS (Hensel *et al*. 1997*b*). The predicted protein SsaV is similar to members the LcrD family of inner membrane transport proteins (Galan *et al*. 1992; Hensel *et al*. 1997*b*), and probably forms a protein-conducting membrane channel of the secreton. The product of *ssaN* has similarity to YscN, a cytoplasmic protein that energizes the *Yersinia* TTSS through its ATPase activity (Woestyn *et al*. 1994). SsaC, encoded by the structural I operon, is similar to InvG (Ochman *et al*. 1996; Shea *et al*. 1996), a member of the PulD family of translocases (Kaniga *et al*. 1994), which is thought to form an outer membrane pore (Crago & Koronakis 1998). Most of the products of the structural I and II operons, together with some proteins encoded by the secretory operon, probably form a secreton that spans the inner and outer membranes of the bacterial cell.

The fourth, secretory operon encodes proteins classed as putative secreted proteins and their cytoplasmic chaperones. The type III secretion apparatus secretes different classes of proteins. The translocon, which is formed by YopB/D in *Yersinia* (Neyt & Cornelis 1999) and IpaB/C in *Shigella* (Blocker *et al*. 1999), forms a pore in the eukaryotic membrane through which further proteins are translocated into the target cell. In the case of SPI-2 this probably involves SseC, which is 24% identical and 48% similar to EspD of EPEC and YopB and has three predicted hydrophobic membrane-spanning domains (Hensel *et al*. 1998). Second, in some secretion systems a protein, such as EspA of EPEC, forms a link between the bacterium and target cell (Ebel *et al*. 1998; Knutton *et al*. 1998), and could act as a channel for the delivery of proteins into the host cell (Frankel *et al*. 1998). SseB of SPI-2 is 25% identical and 47% similar to EspA and its SPI-2-dependent secretion onto the bacterial cell surface has recently been demonstrated (Beuzon *et al*. 1999), making it a likely candidate for this role. Although the functions of several of the structural components of TTSS are conserved in a wide variety of bacteria, those of the effector molecules are quite different. For example the *Yersinia* effectors (YOPS) have a cytotoxic function, whereas the effectors of SPI-1 are involved in epithelial cell invasion (Hueck 1998). The distinction between effector and translocator may, however, be arbitrary: for example IpaC of *Shigella* is a component of the pore formed by the bacterium in the host cell membrane (Blocker *et al*. 1999), but is also involved in activating cytosolic actin polymerization (Tran Van Nhieu 1999). The sequence of SPI-2 does not reveal any candidate effectors such as kinases or phosphatases. However, a gene of the structural I operon, *spiC*, encodes a protein that is translocated by SPI-2 into host cells where it appears to interfere with vacuolar fusion events (Uchiya *et al*. 1999). With the exception of SpiC no other effector proteins of SPI-2 have been identified. It is possible that, as is the case for Inv/Spa, further effector proteins may be encoded outside the pathogenicity island.

Chaperone proteins of the TTSS bind and stabilize secreted proteins in the bacterial cytoplasm. They are small, usually with an acidic pI, and are often encoded upstream of the gene encoding their target. Two predicted proteins of SPI-2 are similar to chaperones of other TTSS: SscA, which is 26% identical to LcrH of *Yersinia pestis*, and SscB, which is 23% identical to IppI of *Shigella* (Hensel *et al*. 1998).

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6. REGULATION OF GENE EXPRESSION

Several environmental factors affect SPI-2 gene expression *in vitro*. Magnesium and phosphate starvation leads to transcriptional activation of a *luc* fusion to *sseA*, the first gene of the secretory operon (Deiwick *et al*. 1999). However, the effect is dependent on growth phase, with maximal activity occurring several hours after these ions are removed. It is therefore likely that phosphate and magnesium starvation are not the expression stimuli *per se* and that other factors present at stationary phase are required for SPI-2 gene expression.

Expression of genes within the TTSS is dependent upon the two-component regulatory system SsrAB. Transcription of an *sseA*::*luc* fusion was reduced 250–300 times in an *ssrA*$^-$ or *ssrB*$^-$ mutant background when bacteria were grown in minimal media (Deiwick *et al*. 1999). The dependence of SPI-2 transcription upon *ssrAB* expression has been confirmed within host cells; transcription of an *ssaH*::*gfp* fusion identified by its 400-fold intracellular induction was not induced within macrophages in strains carrying *ssrA* disruptions (Valdivia & Falkow 1997). Expression of *ssrA* is induced eightfold within mammalian cells soon after entry, and reaches a maximum after 2 h. Although *ssrAB* is able to induce its own expression, it is also affected by other unidentified regulators, because an *ssrA*::*gfp* fusion was induced fourfold in an *ssrA*$^-$ background. Treatment of macrophages with the vacuolar proton ATPase inhibitor bafilomycin A resulted in two- to threefold reduced induction of *ssrAB* expression. An acidic vacuolar pH is therefore required for optimal SPI-2 gene expression in host cells (Cirillo *et al*. 1998).

Although *ssaH* was identified as a macrophage-activated gene, further experiments showed that expression of the *gfp* reporter was induced in a variety of cell types. The *in vivo* relevance of these *in vitro* studies has been confirmed by demonstration of SPI-2 gene transcription in mouse splenocytes and hepatocytes three days post-inoculation (Cirillo *et al*. 1998).

7. FUNCTIONAL ANALYSIS OF THE SPI-2 TTSS

SPI-2 TTSS mutant strains are attenuated by several orders of magnitude when administered orally, intraperitoneally or intravenously to Ity^S or Ity^R mice (Shea *et al*. 1996, 1999). This makes SPI-2 mutants among the most attenuated that have been described in *S. typhimurium*. The attenuation of SPI-2 mutants after intraperitoneal inoculation indicates that the role of the secretion system is at a stage of pathogenesis subsequent to epithelial cell entry. This is in contrast to SPI-1 mutants, which are defective in invasion of the gut epithelium and are thus only attenuated after oral inoculation. After oral infection, SPI-2 mutant strains reach the Peyer's patches but do not persist there and cannot reach the mesenteric lymph nodes, spleen or liver in significant numbers (Cirillo *et al*. 1998). The notion that SPI-2 is important for the establishment or maintenance of systemic infection is supported by the recent work of Tsolis *et al*. (1999), who found that, whereas SPI-1 mutants are unable to cause disease in the calf diarrhoea model, SPI-2 mutants are not significantly attenuated.

Although SPI-2 mutant strains have no obvious morphological defects and have similar growth rates to wild-type strains in minimal and rich media, work from several laboratories has established that they fail to accumulate in a variety of host cells, including RAW and J774 macrophages (Cirillo *et al*. 1998; Hensel *et al*. 1998; Ochman *et al*. 1996; Uchiya *et al*. 1999), elicited peritoneal macrophages (Hensel *et al*. 1998), and Hep-2 and HeLa epithelial cells (Cirillo *et al*. 1998). SPI-2 mutants are recovered in lower numbers than wild-type from the mesenteric lymph nodes, liver and spleen during systemic infection (Cirillo *et al*. 1998; Shea *et al*. 1999). However, since the primary sites of *S. typhimurium* replication *in vivo* are splenic macrophages and liver Kupffer cells (Richter-Dahlfors *et al*. 1997), these are most probably the cells in which the SPI-2 secretion system mediates its effect *in vivo*. *In vivo* time-course experiments in BALB/c mice reveal an approximate tenfold increase in the numbers of SPI-2 mutants between 4 and 24 h following intraperitoneal inoculation. The bacterial load then remains relatively static for several days before being cleared. This failure of mutant strains to accumulate further could be the result of either a reduced ability of the bacteria to replicate or an increased susceptibility to the bactericidal defences of the host, or both. It is possible to distinguish between these two possibilities *in vivo* by measuring the dilution of a temperature-sensitive plasmid (pHSG422) within the bacterial population during infection (Benjamin *et al*. 1990; Gulig & Doyle 1993). This plasmid cannot replicate at 37 °C and becomes diluted in the population as the bacteria divide. The total number of bacteria and the proportion of these carrying the plasmid are counted by plating the inoculum and spleen homogenates from infected mice with the appropriate antibiotics. If the difference between the total numbers of wild-type and mutant strains is due to an increased susceptibility of the mutants to killing, the number of mutant cells carrying the plasmid will be lower than the number of plasmid-carrying wild-type cells. If, however, there is no significant difference in sensitivity to killing but the mutants are unable to replicate efficiently, the numbers of each strain carrying the plasmid should be approximately equal (figure 2). After a 16 h infection of wild-type and SPI-2 mutant strains in mice, the numbers of each strain carrying the non-replicating plasmid were approximately equal in spleen homogenates, although there were in total tenfold more wild-type than mutant cells (Shea *et al*. 1999). This indicates that SPI-2 enables growth of the bacterial population rather than conferring resistance to host killing mechanisms. It also showed that by 16 h, wild-type cells of the inoculum had undergone five or six divisions whereas the SPI-2 mutant cells had undergone approximately half as many divisions. Recent work suggests that the failure of mutants to accumulate intracellularly is connected with an inability to modify trafficking of the *S. typhimurium* phagosome (Uchiya *et al*. 1999).

A characteristic of *S. typhimurium* growth in host cells is its ability to replicate in a specialized phagosome, the *Salmonella*-containing vacuole (SCV), which avoids interacting with lysosomal compartments. SPI-2 could secrete and translocate factors into the cytosol to alter the trafficking pathways of the host cell (figure 3). Uchiya *et al*. (1999) found that the SPI-2-encoded protein SpiC is secreted into the cytosol of J774 macrophages 1–6 h after bacterial uptake, but only by strains of *S. typhimurium* with

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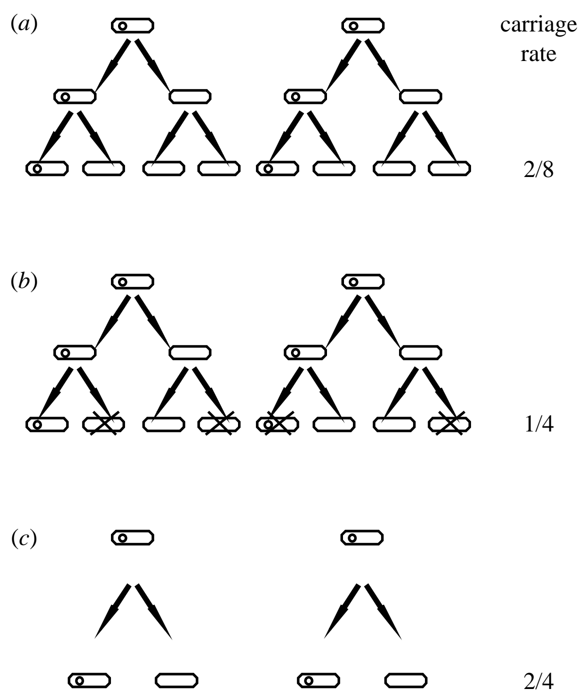

Figure 2. Use of a temperature-sensitive non-replicating plasmid to measure relative growth and killing rates of *S. typhimurium* in the mouse. (*a*) The dilution of the plasmid in the wild-type population is proportional to the number of rounds of bacterial cell division. (*b*) If a mutant strain has increased sensitivity to host killing mechanisms there will be lower bacterial numbers and fewer plasmid-carrying cells than for the wild-type strain. (*c*) If a mutant strain has a decreased ability to divide normally in the host, there will be lower bacterial numbers but the same number of plasmid-carrying mutant cells as in the wild-type strain. Adapted from Gulig & Doyle (1993).

a functional SPI-2 secretion system. Infection with wild-type *S. typhimurium* reduces the overall rate of phagosome–lysosome fusion within the cell, as demonstrated by co-localization of bovine serum albumin (BSA)-gold with lysosomal markers. *spiC* mutants do not have this characteristic, and trafficking of BSA-gold in macrophages infected with these strains is similar to that of uninfected J774 cells.

If the function of SPI-2 is to restrict interactions between the SCV and toxic lysosomal compartments, we might expect to see increased killing of SPI-2 mutants compared with wild-type cells *in vivo*. However, the experiments with the non-replicating plasmid *in vivo* described above show that there is little difference in the degree of killing sustained by the two strains and that the defect of SPI-2 mutants is related to their inability to divide at a normal rate. Perhaps SpiC is therefore involved in altering SCV trafficking which enables intracellular *S. typhimurium* to obtain nutrients or other factors necessary for growth and replication inside the vacuole, rather than avoiding killing by lysosomal enzymes or other host cell mechanisms.

8. REGULATION OF SECRETION

The SCV undergoes acidification to pH 4.0–5.0 within 1 h after bacterial uptake (Rathman *et al.* 1996). Beuzon *et al.* (1999) investigated whether secretion of a SPI-2 protein *in vitro* could be induced by growth in media simulating the conditions within the vacuole. The secreted protein studied, SseB, is similar to EspA of EPEC, which is a component of a filamentous surface appendage forming a contact between the bacterium and the eukaryotic cell (Ebel *et al.* 1998; Knutton *et al.* 1998). SseB is secreted onto the surface of the cell, from which it can be removed by extraction with hydrophobic agents that allow recovery of proteins weakly associated with the bacterial cell surface but do not remove known membrane proteins. This secretion was shown to be dependent on a functional TTSS, and occurred only at pH 4.5–5.0 after growth to stationary phase in minimal media. The induction of secretion was independent of expression, as there was no difference in intracellular levels of SseB at pH 5.0 and 7.5, and SseB was not secreted at pH 7.5 when expressed from a constitutive promoter (Beuzon *et al.* 1999). In *Yersinia*, regulation of secretion involves the protein YopN, which appears to act as a plug blocking the secretion pore until it is itself secreted. SPI-2 does not contain a homologue of *yopN* but some further control mechanism must exist because SseB is expressed but not secreted in minimal media at neutral pH, and is secreted shortly after a shift to acidic media, indicating that the secreton is probably assembled before the pH shift.

Little is known about the regulation and timing of secretion by SPI-2 *in vivo*. However, SpiC was detected inside infected macrophages within 1 h of entry (Uchiya *et al.* 1999). This is difficult to reconcile with the findings of Cirillo *et al.* (1998), who showed that expression of the SPI-2 regulatory and secretory apparatus genes does not increase greatly until 2 h post-uptake, and reaches a maximum after 6 h. Although SseB is immediately secreted onto the cell surface after acidification of the culture media, this is presumably conditional on the presence of a preformed secretion apparatus. Expression of SPI-2 genes *in vitro* requires low magnesium and phosphate and occurs as cells enter the stationary phase of growth. However, it is not known at what stage of *in vivo* infection the secretion apparatus is assembled. Further work is therefore needed to determine the kinetics of SPI-2 secretion within host cells *in vivo*.

9. RELATIONSHIPS BETWEEN VIRULENCE FACTORS *IN VIVO*

In a mixed infection of BALB/c mice, the ability of two *S. typhimurium* strains to colonize the spleen provides a measure of their relative virulence. The CI, defined as the output ratio of mutant to wild-type bacteria divided by the input ratio of mutant to wild-type bacteria, is a quantitative value for the degree of attenuation of a mutant strain, with the CI of a wild-type strain versus a fully virulent derivative being approximately 1.0.

A further development of this method in our laboratory is its use to distinguish whether two genes have the same biochemical function *in vivo*. This is achieved by creating a double mutant strain, which is combined in a mixed inoculum with a strain carrying only one of the mutations. The attenuation caused by the mutation present in both strains will be equivalent, so the CI calculated from this experiment will reflect the effect of the second mutation in the absence of the first gene. If the functions of the two

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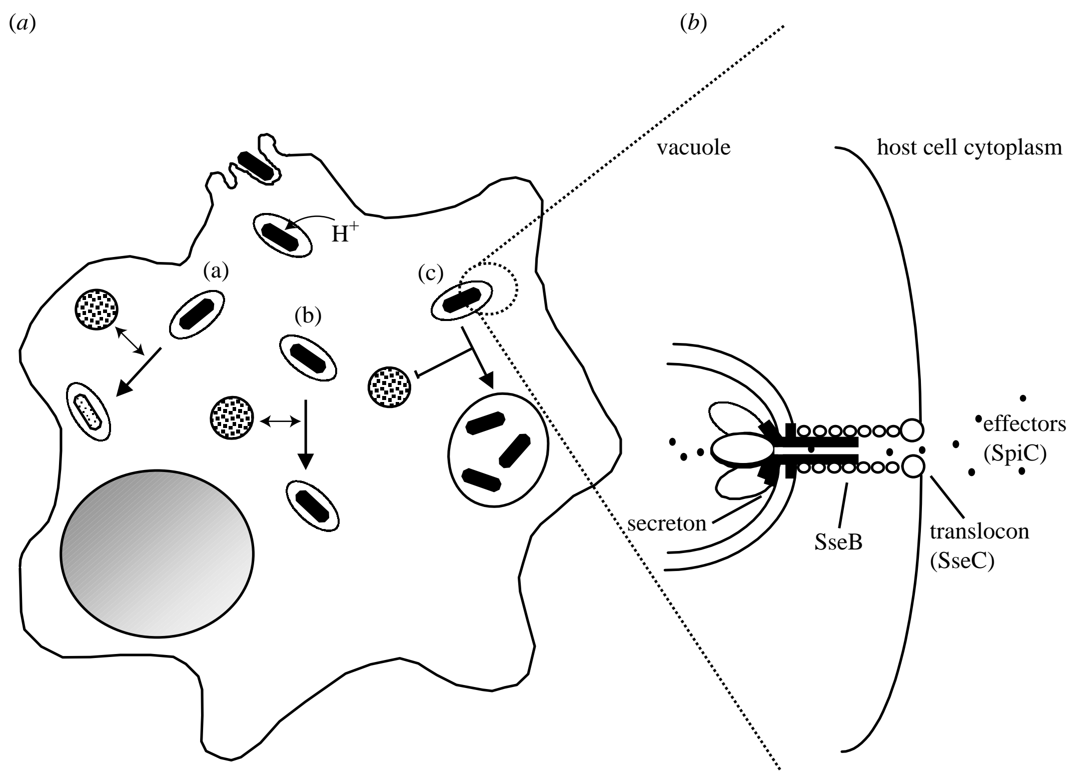

Figure 3. (*a*) Putative trafficking of *S. typhimurium* within the macrophage. After entry, the SCV undergoes acidification (Rathman *et al.* 1996). (a) Phagosomes containing heat-killed bacteria interact with lysosomal compartments and are ultimately degraded (Rathman *et al.* 1997). (b) SCVs containing SPI-2 mutant strains interact with lysosomal compartments (Uchiya *et al.* 1999). *In vivo* data suggest that this does not result in an increased rate of killing but the bacteria are unable to replicate to the levels of wild-type cells (Shea *et al.* 1999). (c) A proportion of wild-type *S. typhimurium* SCVs do not interact with lysosomal compartments (Rathman *et al.* 1997). They form a specialized vacuole in which bacterial replication occurs. (*b*) Putative structure of the SPI-2 TTSS. A link between the bacterium and host vacuolar membrane is formed by SseB. SseC forms a pore in the vacuolar membrane for translocation of effectors, including SpiC (Uchiya *et al.* 1999), into the host cell cytoplasm.

genes are totally independent, the CI will be equal to the CI of a strain carrying only the second mutation versus the wild-type strain, and the CI of the double mutant against the wild-type strain should reflect the effects of the two mutations in an additive manner. If the two genes are involved in the same biochemical function, no additional attenuation should be conferred on a single mutant strain by the acquisition of the second mutation, and the predicted CI of the double mutant versus the single will be approximately 1.0. In a situation where the functions of the two genes partially overlap the CI may be intermediate. However, because the function of one virulence gene may be dependent on the function of another biochemically unrelated gene, it is difficult to interpret a phenotype that is not completely additive. The lack of further attenuation by the presence of a second mutation may not necessarily mean that the functions of two genes are directly related, but simply that the attenuation caused by one mutation prevents the infection process reaching the stage when the second gene acts. Manipulation of the animal model (by varying the inoculum dose or route of infection, for example) may allow the effects of the second mutation to be observed. If introduction of a second mutation increases attenuation in a completely additive fashion, it can be concluded that the genes in question are not related *in vivo*.

It has been suggested that the *spv* genes, encoded on the *S. typhimurium* virulence plasmid, may be related to SPI-2 because of the striking similarities of their mutant phenotypes (Shea *et al.* 1999). SPI-2 and *spv* mutant strains have a similar level of attenuation in the mouse, which cannot be transcomplemented *in vivo* by the presence of virulent strains. Both sets of genes are transcribed within host cells, and mutants are defective for intracellular survival, the defect being related to bacterial growth rate, not an increased susceptibility to killing. However, the ability of an $ssaV^{-}$, $spvA^{-}$ strain to colonize the spleen in competition with an $ssaV^{-}$ strain was not statistically different from the ability of an $spvA^{-}$ strain to colonize the spleen in competition with a wild-type strain (table 2) (Shea *et al.* 1999). The attenuation conferred by these two mutations is therefore entirely additive, indicating that they most probably have completely independent functions.

Many aspects of pathogenesis and survival in the host cannot be simulated in a test tube or cell culture model, and artificial factors that do not exist in a natural infection may complicate the interpretation of *in vitro* results. As a result of these limitations, a mutant phenotype observed in an *in vitro* assay does not necessarily mean that this is relevant *in vivo*. Where different *in vitro* techniques have produced conflicting results, it is especially important to try to obtain *in vivo* evidence to clarify the

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Table 2. *CI values for mixed infections of* S. typhimurium *strains in mice*

(Mice were inoculated intraperitoneally with approximately 10^5 colony-forming units (CFU) of a mixed inoculum. Mouse spleens were harvested after 48 h for enumeration of bacterial CFU. The strains used were differentiated on the basis of antibiotic sensitivity. The CI was calculated as the output ratio of strain 2 to strain 1 divided by the input ratio of strain 2 to strain 1. Adapted from Shea *et al*. (1999).)

strain 1	strain 2	CI
12023	*ssaV*$^-$	0.058
ssaV$^-$	*ssaV*$^-$	1.14
ssaV$^-$	*ssaV*$^-$, *sseC*$^-$	1.28[a]
12023	*purD*$^-$	0.005
ssaV$^-$	*ssaV*$^-$, *purD*$^-$	0.008[b]
12023	*spvA*$^-$	0.068
ssaV$^-$	*ssaV*$^-$, *spvA*$^-$	0.052[b]

[a]Not significantly different ($p > 0.5$) from the CI obtained for the mixed infection *ssaV*$^-$ versus *ssaV*$^-$.
[b]Significantly different ($p > 0.05$) from the CI obtained for the mixed infection *ssaV*$^-$ versus *ssaV*$^-$.

situation. A case in point is the contribution of the wide domain regulator PhoPQ to regulation of SPI-2 gene expression. An *ssaH::gfp* fusion did not require a functional *phoP* gene for intramacrophage induction (Valdivia & Falkow 1997). Furthermore, Cirillo *et al*. (1998) found that intramacrophage SPI-2 gene expression was reduced by bafilomycin, whereas PhoPQ-dependent expression is not affected by this drug (Rathman 1996), and it was concluded from this that SPI-2 expression is independent of PhoPQ. However, Deiwick *et al*. (1999) reported that levels of *in vitro* SscA expression were greatly reduced in a *phoP*$^-$ strain. In our laboratory, *in vivo* competition experiments involving a *phoPQ* mutant and a *phoPQ*, SPI-2 double mutant have failed to provide any evidence that these two systems interact in the host, because the CI of this combination was similar to that of a SPI-2 mutant against the wild-type strain, and the CI of the double mutant against the wild-type strain reflected a completely additive effect on attenuation of the two mutations (C. Beuzon, unpublished data).

10. CONCLUDING REMARKS

A variety of genetic approaches are now available to study the behaviour of bacterial pathogens *in vivo*. STM allows a mutation-based screen for genes that contribute to pathogenesis, which has been applied successfully to a variety of pathogens including *S. typhimurium*. Where the functions of genes identified by this and other approaches are not clear from analysis of their nucleotide sequence, further analysis of the mutant strains *in vitro*, and the use of the CI test and plasmid segregation studies, can provide important information about the functions and interactions of these genes *in vivo*. It is worth noting that, in the case of *S. typhimurium* SPI-2 TTSS, the results of some *in vitro* studies are not in accord with the phenotypes of mutant strains *in vivo*, and should therefore be interpreted with caution, until more sophisticated methods are available to study gene function *in vivo*.

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