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Review Article

Identification of bacterial genes required for in-vivo survival

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Abstract

Genetic approaches used for in-vivo studies of bacterial pathogenesis are providing insights into how bacteria disrupt host defences and exploit host molecules for their own advantage. Signature tagged mutagenesis (STM) provides a means of identifying the genes involved in the process of infection, particularly those genes that are important for bacterial proliferation in-vivo. In this review, the application of STM to the understanding of bacterial pathogenesis and findings from work on three human pathogens, *Salmonella typhimurium*, *Mycobacterium tuberculosis* and *Neisseria meningitidis*, are discussed. The next challenge is to understand how these and other genes influence the infective process at the molecular and cellular levels and to design novel interventions to block the progression of disease.

Introduction

Bacterial pathogens have evolved sophisticated mechanisms to sense and respond to specific environmental cues provided by the host during pathogenesis (Finlay & Falkow 1989). The genetic basis of how microbes coordinate the expression of genes during the disease process has been studied intensively and the findings indicate the importance of the micro-environment in influencing the behaviour of bacteria during the transition from harmless commensal to invading pathogen. Recognition of the crucial role of this interplay, in which microbes respond to specific cues provided by the host, emphasizes the need to perform studies on pathogenesis in-vivo (Smith 1998).

Recently, methodologies have been developed that enable genetic approaches to be used for in-vivo studies of bacterial pathogenesis (Mahan et al 1993; Hensel et al 1995), and the results have provided important insights into the nature of how bacteria subvert host defences and exploit host molecules for their own ends. The approaches can be broadly divided into those that pinpoint genes that are specifically switched on in the host, and those that define genes that are indispensable for the invading bacterium during pathogenesis (Hensel & Holden 1996). In-vivo expression technology and differential fluorescence induction (Mahan et al 1993; Valdivia & Falkow 1997) were designed to identify genes that are expressed only during pathogenesis, whereas signature tagged mutagenesis (STM) allows mutational analysis to be performed in-vivo (Hensel et al 1995). STM has several distinct advantages over the transcriptional approaches to gene identification. In mutational analysis, selection is based on the behaviour of the bacterium in the host rather than just the profile of gene expression; the relationship between an alteration in the transcriptional activity of a gene in a given environment and its function is not secure (Winzeler et al 1999). Mutational analysis is a more rigorous

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way of determining gene function than transcriptional analysis, and provides researchers with strains harbouring single, genetically defined deletions that are a powerful resource for further characterization. In this paper, we outline how STM has been applied to the understanding of bacterial pathogenesis, and discuss findings from work on three important human pathogens: *Salmonella typhimurium*, *Mycobacterium tuberculosis* and *Neisseria meningitidis*. The biological significance and potential applications from the results are discussed.

Signature tagged mutagenesis

STM was originally devised by David Holden while studying the filamentous fungus, *Aspergillus fumigatus*. At that time, his group were attempting to define virulence determinants for this opportunistic pathogen by selecting candidate genes (Tang et al 1993; Smith et al 1994). Isogenic deletion mutants were constructed in selected genes and each mutant was tested individually in animal models of infection. As this approach drew more and more frustrating blanks, STM was devised to allow high-throughput, unbiased genetic screening of libraries of mutants in-vivo. By tagging each mutant with a unique 40-bp sequence, it became possible to differentiate individual mutants from each other within a mixed population. Multiple mutants could therefore be examined simultaneously for their ability to cause sustained infection in a single animal (Hensel & Holden 1996). The tagging was achieved by engineering the sequence identifiers into a transposon, a commonly used tool for making insertional mutations in bacteria. The tagged transposons were then delivered into the pathogenic strain to generate large numbers of individually marked mutants. This paved the way for large-scale screening of libraries of mutants in biologically relevant animal models of disease, rather than relying on experiments confined to the laboratory. STM has now been successfully applied to a wide number of bacterial and fungal pathogens (Mei et al 1997; Polissi et al 1998; Cormack et al 1999; Darwin & Miller 1999; Lestrate et al 2000; Martindale et al 2000).

Salmonella typhimurium

S. typhimurium was used for proof-in-principle of STM because of its excellent genetic systems and well-validated animal models. *S. typhimurium* causes gastroenteritis in humans, but, in mice, it leads to systemic infection after oral ingestion with spread of the bacterium to cells of the reticulo-endothelial (RE) system. Therefore, murine infection with *S. typhimurium* replicates human typhoid fever. Typhoid is endemic in many parts of the developing world and the emergence of multiply resistant strains has made this infection increasingly difficult to treat (Sood et al 1999). The mechanisms underlying the entry of *Salmonella* into gastrointestinal epithelial cells had been extensively studied in-vitro. This work revealed the existence of a large segment of the chromosome, the *Salmonella* pathogenicity island 1 (SPI-1), which contains a cluster of virulence genes (Mills et al 1995; Hacker et al 1997), including those encoding a type three secretion system (TTSS). A wide range of Gram-negative pathogens exploit TTSSs to inject their own proteins into host cells, often leading to cytoskeletal changes. Depending on effectors, this can mediate bacterial adhesion, entry or antiphagocytosis (Finlay & Cossart 1997).

In the original STM screen, tagged *S. typhimurium* mutants were inoculated into mice and examined for their ability to cause systemic infection. The work identified a number of known *S. typhimurium* virulence determinants as well as novel sequences that were subsequently shown to form part of a second TTSS, encoded on a new pathogenicity island, SPI-2 (Shea et al 1996). SPI-2 was only identified through its role in disseminated infection, having been overlooked during investigation of epithelial cell entry in-vitro, and thus its biological function is entirely distinct from SPI-1. SPI-2 genes have since been shown to be involved in the survival of *Salmonella* within macrophages, a component of the RE system (Ochman et al 1996; Hensel et al 1998). Precisely how SPI-2 promotes bacterial survival within macrophages is still not fully understood, as SPI-2 gene products appear to subvert several host mechanisms for eliminating *Salmonella*. SPI-2 appears to be involved in avoidance of NADPH oxidase-dependent killing (Vazquez-Torres et al 2000), and in the trafficking of the bacterium to privileged compartments within the host cell (Uchiya et al 1999; Beuzon et al 2000).

The realization that SPI-2 is required for systemic disease, but not for localized gastrointestinal infection, led to the idea that SPI-2 mutations might be included in live attenuated *Salmonella* vaccines. *S. typhimurium* strains harbouring a mutation in either of two SPI-2 genes, *sseC* and *sseV*, have been successfully used to deliver heterologous antigens to mice (Medina et al 1999). More recently, studies have been conducted in human volunteers to further test the feasibility of using strains with SPI-2 deletions as *S. typhi* vaccines. The limited available data suggests that the strains are immunogenic and well tolerated (David Lewis, personal communication), and further studies are awaited.

Therefore, within a short space of time, STM in *Salmonella* has led from proof-in-principle all the way to potential clinical applications.

Mycobacterium tuberculosis

M. tuberculosis is the largest single cause of death owing to an infectious disease in the world. The only vaccine currently available against *M. tuberculosis* is Bacille Calmette-Guerin (BCG), a live attenuated strain of *Mycobacterium bovis* that was originally obtained by serial passage in the laboratory (Andersen 2001). However, BCG is only partially effective in preventing *M. tuberculosis* infections, and new vaccines are urgently required given the continued impact of this disease in human populations, through the emergence of multiply resistant strains and the advent of the HIV pandemic, resulting in a new group of highly susceptible individuals (Zumla et al 1999). Further understanding of how the bacterium is able to infect and survive within the human lung (the primary site of tuberculosis) could be extremely valuable in the rational design of new TB vaccines. STM has been performed on *M. tuberculosis* by two groups who examined the bacterium's ability to cause pulmonary disease (Camacho et al 1999; Cox et al 1999).

To screen the mutant libraries, animals were intravenously inoculated with *M. tuberculosis* mutants, and three weeks later bacteria were recovered from the lungs. Both groups found attenuating insertions in a 50-kb virulence gene cluster that contains 13 open reading frames. Seven open reading frames appear to be involved in the biosynthesis of components of the mycobacterial cell envelope, phthiocerol, phenolphthiocerol and mycoside B (Camacho et al 1999; Cox et al 1999). These lipid-based molecules are largely restricted to pathogenic mycobacteria, having been previously found in eight mycobacterial species, seven of which are pathogenic (Daffe & Laneelle 1988).

Analysis of the lipid content of the mutants was used to further characterize the function of genes in this region (Cox et al 1999; Camacho et al 2001). Three mutants with defects in the synthesis or export of phthiocerol dimycoserosate grew as well as the wild-type bacterium in the liver and spleen, but had clear defects for survival in the lung. The results provide fascinating insights into the requirements for growth in different host environments, and how cell-surface lipids of *M. tuberculosis* influence its ability to survive within the lung (Cox et al 1999). Furthermore, it demonstrates how STM can be adapted to reveal the molecular basis of the tropism for host niches exhibited by pathogenic microbes.

The work on *M. tuberculosis* may lead in several directions. For studies on pathogenesis, it is still unclear how these lipid molecules confer advantages to the microbe within the lung. Do the lipid components of the *M. tuberculosis* cell wall act as adhesins or do they enhance the bacterium's ability to withstand killing by innate immune mechanisms? In terms of clinical applications, this class of molecules could act as the target for novel antimycobacterial drugs or be deleted in live attenuated strains, which could replace the BCG vaccine.

Neisseria meningitidis

N. meningitidis is a Gram-negative diplococcus that is a part of the normal nasopharyngeal flora in up to 20% of healthy individuals (Cartwright et al 1987). Occasionally, the bacterium gains access to the systemic circulation through mechanisms that are not fully understood, and establishes a disseminated infection with devastating consequences. Bacteraemia is required for all the pathological sequelae of meningococcal infection including septicaemia and meningitis (Herrick 1918), and is therefore a key step in meningococcal pathogenesis. Serogroup-specific vaccines are available to prevent serogroup A and C infections, but there is still no vaccine against serogroup B, the leading cause of meningitis in developed countries. We have recently performed STM on *N. meningitidis* serogroup B in order to isolate bacterial determinants involved in septicaemic disease (Sun et al 2000).

An important constraint of STM is the need to generate libraries of diverse insertional mutants in the organism of interest, a significant problem for work with *Neisseria* spp. (Claus et al 1998). Mutagenesis was successfully performed using a recently developed method in which *Neisseria* DNA was modified in-vitro using the purified components of Tn*10*, a widely used bacterial transposon (Chalmers & Kleckner 1994). *N. meningitidis* efficiently takes up exogenous DNA and integrates it by homologous recombination, so the modified genes were then introduced into *N. meningitidis* by natural transformation. The mutants were then screened for their ability to cause systemic infection in the neonatal rat model. This model has previously been used to study the influence of lipopolysaccharide, capsule and iron acquisition on pathogenesis (Stojiljkovic et al 1995; Vogel et al 1996), and the protective efficacy of antibodies against outer membrane proteins (Saukkonen et al 1987). STM of *N. meningitidis* led to the identification of 73 genes required for bacteraemic disease (Sun et al 2000). Eight insertions were in genes

encoding known pathogenicity factors, while the remainder have not been previously implicated in *N. meningitidis* virulence, and therefore provide novel insights into the molecular mechanisms underlying meningococcal infection. For instance, a significant proportion (around 20%) of genes identified were of previously unknown function, and some of these are highly conserved across bacterial genera. These may form the targets for compounds for treating a broad spectrum of bacterial infections. Furthermore, a number of genes were identified that are required for the biogenesis of known drug and candidate vaccine targets. For instance, several independent attenuating insertions were found in genes encoding enzymes in the aromatic amino acid and purine biosynthetic pathways. Both these pathways have been targeted for antimicrobials and in live attenuated vaccines.

The available whole genome sequences of two strains of *N. meningitidis* allowed the genomic distribution of attenuating mutations to be mapped (Parkhill et al 2000; Tettelin et al 2000). Clusters of virulence genes were observed, most notably those involving iron uptake. Also, it was simple to predict insertions that may have effects on downstream genes leading to the identification of operons that may be important for pathogenesis. The results provide the first comprehensive analysis of the attributes of *N. meningitidis* that enable it to cause invasive infection, and further analysis of the genes identified by this study may lead to the development of vaccines for the prevention of meningococcal infection.

Summary

In recent years, there has been a resurgence in the threat posed by infectious diseases. In addition, some newly emerging infections are now resistant to all conventional anti-microbial agents, and therefore new classes of antibiotics and vaccines are urgently needed to protect human populations from bacterial pathogens (Levy 1998). The complete nucleotide sequences are now available for most bacterial pathogens, providing a comprehensive catalogue of their genetic composition. However, a striking and common feature of the genomes are the large proportion of genes (up to 40%) that are still of unknown function (Strauss & Falkow 1997; Tang et al 1997); studies on bacteria in the laboratory for 50 years have failed to reveal the identity and role of these open reading frames. Recent genetic methods, such as STM, now allow high-throughput analysis of bacteria in in-vivo environments, and have shown that some of these genes are involved in pathogenesis. The major challenge is now to understand how these and other genes influence the infective process at molecular and cellular levels, so that novel interventions can be designed to block disease progression.

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