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Measurement of bacterial gene expression *in vivo*

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The complexities of bacterial gene expression during mammalian infection cannot be addressed by *in vitro* experiments. We know that the infected 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. This response involves hundreds of *ivi* (*in vivo*-induced) genes which have recently been identified in animal and cell culture models using a variety of technologies including *in vivo* expression technology, differential fluorescence induction, subtractive hybridization and differential display. Proteomic analysis is beginning to be used to identify IVI proteins, and has benefited from the availability of genome sequences for increasing numbers of bacterial pathogens. The patterns of bacterial gene expression during infection remain to be investigated. Are *ivi* genes expressed in an organ-specific or cell-type-specific fashion? New approaches are required to answer these questions. The uses of the immunologically based *in vivo* antigen technology system, *in situ* PCR and DNA microarray analysis are considered. This review considers existing methods for examining bacterial gene expression *in vivo*, and describes emerging approaches that should further our understanding in the future.

Keywords: green fluorescent protein (GFP); *in vivo* expression technology; *in vivo* induced fluorescence (IVIF); fluorescence-activated cell sorting; *in vivo* antigen technology; Lux

1. INTRODUCTION

Understanding the expression of genes involved in infection remains the holy grail for research into bacterial virulence. Over the past three decades, research has focused on bacteria grown in monoculture in laboratory media to characterize the responses to many environmental stimuli. The simultaneous development of molecular biological technology and molecular genetic approaches has facilitated the dissection of a plethora of regulatory systems (Mekalanos 1992). The scene has been set for the investigation of patterns of gene expression in more complex systems and the consideration of the bacterial response to the host environment *in situ*. It is likely that this will reveal fascinating and novel bacterial responses, as well as new hierarchies of genetic regulation (Hinton 1997). In this review we describe the current tools for the analysis of bacterial gene expression *in vivo*, their limitations and some promising new approaches.

2. APPROACHES FOR ASSESSING PATTERNS OF *IN VIVO* GENE EXPRESSION

(a) *Reporter systems for indirect monitoring of bacterial mRNA levels* in vivo

Bacterial gene expression is assessed by the direct or indirect measurement of mRNA levels. Reporter genes are used to monitor transcription indirectly by putting genes that encode an assayable protein under the control of promoters of interest. Various reporter gene products have been used for molecular genetic analyses, and they all share the ability to glow, to fluoresce or to be assayed colorimetrically (Miller 1992; Sala-Newby *et al.* 1999). The increased use of reporter systems that do not naturally occur in most bacterial or mammalian cells has improved the sensitivity of monitoring bacterial gene expression *in situ*. These recent developments now allow gene expression to be studied not only in large bacterial populations but also in individual bacterial cells.

(i) *β-galactosidase*

The reliable β-galactosidase reporter system has been extensively used to monitor gene activity in response to various environmental conditions (Casadaban & Cohen 1979; Jacob & Monod 1961; Silhavy & Beckwith 1985). The chromogenic substrate nitrophenyl-β-D-galactopyranoside has been used for simple and accurate quantification of β-galactosidase activity for cultures grown *in vitro*. Increasingly, β-galactosidase is being used for analysis of gene expression *in vivo*; for example, expression of the *spvB* virulence gene of *Salmonella typhimurium* was monitored with a β-galactosidase reporter system in cultured macrophages and non-phagocytic cells (Fierer *et al.* 1993). The activation of *Salmonella* PhoP-regulated genes was assessed in phagosomes within macrophages with β-galactosidase transcriptional fusions (Aranda *et al.* 1992). However, a major limitation of the β-galactosidase reporter system is that it is invasive, requiring permeabilization of target bacterial cells. Because the efficiency of cell permeabilization varies, observed differences in β-galactosidase activity can reflect altered substrate uptake rather than

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changing levels of gene expression, preventing accurate analysis of individual bacterial cells (Nwoguh *et al.* 1995). Furthermore, studies involving animal and cell models require the isolation of the bacteria prior to β-galactosidase assay. Despite the development of sensitive fluorogenic substrates for the quantification of gene expression (Garcia-del-Portillo *et al.* 1992; Handfield *et al.* 1998; Heithoff *et al.* 1997; Slauch *et al.* 1994), and a new electrochemical approach for online monitoring of β-galactosidase activity (Biran *et al.* 1999), robust assessment of the kinetics of gene expression *in vivo* requires other approaches.

(ii) *Luciferases*

Luciferases isolated from fireflies or bioluminescent bacteria (LuxAB from *Vibrio* spp.) have been popular as reporters for gene expression in a variety of organisms including Gram-negative and Gram-positive bacteria, yeast, plant cells and transgenic plants (Kirchner *et al.* 1989; Langridge *et al.* 1991*a*,*b*; Meighen 1991; Schauer 1988; Stewart & Williams 1992).

Several reasons have been used to justify the choice of luciferase as a reporter for gene expression. (i) LuxAB has a short half-life, ensuring that photon production reflects real-time gene expression. This can be advantageous, but does make the system sensitive to experimental pertubations. (ii) The signal produced by luciferase is ideal for gene expression studies because low levels of light can be measured and linearly quantified over several orders of magnitude. For example, a comparison of the LuxAB and β-galactosidase reporter systems to assess *spvB* gene expression from *S. typhimurium* inside mammalian cells showed that light emission mediated by Lux was a faster and more easily detectable signal than β-galactosidase activity (Pfeifer & Finlay 1995). (iii) As luciferase enzymes are not widespread in bacterial pathogens, endogenous background does not present a problem.

Three examples demonstrate the value of the *lux* reporter; the mini-Mu derivative, Mu*dlux* was used to assess the response of *S. typhimurium* to adhesion to cultured macrophages (Francis & Gallagher 1993). In *Yersinia pseudotuberculosis*, *luxAB* fusions showed that *yopE* and *yopH* expression was highest during early stages of colonization of either Peyer's patches or the spleen of infected mice (Forsberg & Rosqvist 1994) and a *yopE:lux* fusion was used to demonstrate that *yopE* was induced by contact with HeLa cells (Pettersson *et al.* 1996). However, the toxicity of the aldehyde substrate of LuxAB and of the intracellular expression of luciferase in enterobacteria (Gonzalez-Flecha & Demple 1994) and the variation of Lux activity with oxygen concentration has led the reliability of Lux for measurement of bacterial gene expression *in vivo* to be questioned, particularly in low oxygen environments (Camilli 1996). A further limitation of Lux as a reporter gene is that the photon yield of luciferase does not permit visualization of gene expression in individual bacterial cells.

Lux has been used for the detection of bacterial pathogens such as *Mycobacterium smegmatis* during infection (Sarkis *et al.* 1995). Contag *et al.* (1995) used the Lux operon derived from *Photorhabdus*, which does not require an exogenous substrate, to develop a method for non-invasive optical monitoring of bacterial infections in whole animals. *Salmonella* bacteria that expressed high levels of Lux were visualized within a live mouse with an intensified charge-coupled-device (CCD) camera. Despite photon absorption by mouse organs and skin, bacteria were seen in many infected tissues, including the caecum, spleen and liver. This promising approach has not yet been applied to direct monitoring of gene expression in whole animals.

(iii) *Green fluorescent protein*

Experiments addressing real-time or *in situ* measurements of gene expression tend to use fluorescence-emitting reporter systems, which have the greatest sensitivity. All of these require exogenous substrates, except the 238 residue green fluorescent protein (GFP) protein from *Aequoria* jellyfish, which naturally emits green fluorescence after excitation by blue light (Cubitt *et al.* 1995; Prasher *et al.* 1992; Ward 1998). Because investigation of gene expression *in vivo* requires non-invasive reporters, GFP-based systems have several advantages, particularly when compared to β-galactosidase and luciferase (Jaiwal *et al.* 1998). GFP does not require the addition of any substrate or co-factor. It is a small protein remaining fluorescent even when fused to proteins of interest, making it ideal for studying protein translocation. GFP allows detection and quantification of bacterial gene expression in tissue culture models and promises real-time visualization of gene expression in different pathogens. Expression of GFP does not cause attenuation or reduce bacterial invasion of cultivated cell lines (Valdivia & Falkow 1997*a*; Zhao *et al.* 1998).

Initially, the use of *gfp* as a reporter gene of bacterial promoter activity was hampered by technical limitations such as weak fluorescence intensity and formation of inclusion bodies following over-expression (Valdivia *et al.* 1998). Brighter, more soluble and blue-shifted mutants of GFP obtained by DNA shuffling or oligo-directed mutagenesis (Cormack *et al.* 1996; Crameri *et al.* 1995; Heim *et al.* 1994; Heim & Tsien 1995; Siemering *et al.* 1996) are suitable for *in situ* analyses (Chalfie *et al.* 1994).

GFP has been used for a range of different purposes including use as a transfection marker in eukaryotic cells, such as *Schizosaccharomyces pombe* (Atkins & Izant 1995). Microbial ecological applications include the use of GFP as a bacterial identification marker (Eberl *et al.* 1997). GFP has had considerable impact in localizing proteins within bacteria or after translocation into the cytosol of the host cell (Chen *et al.* 1999; Jacobi *et al.* 1998; Weiss *et al.* 1999). Increasing use is being made of GFP as a reporter of gene activity in various *in vivo* models, such as cultivated cell lines (*S. typhimurium*, Valdivia *et al.* 1996; Falkow 1997; *Y. pseudotuberculosis*, Valdivia *et al.* 1996; *Mycobacterium* spp., Parker & Bermudez 1997; Dhandayuthapani *et al.* 1995; Kremer *et al.* 1995) or in an animal model (*Mycobacterium marinum*, Barker *et al.* 1998). GFP reporters have also been used to investigate regulation of gene expression in *S. typhimurium* and *Candida albicans* (Cirillo *et al.* 1998; Morschäuser *et al.* 1998). The limitations of GFP as a reporter of gene expression are discussed in § 2(c)(ii).

(b) *Analytical techniques for measuring gene expression* in vivo

The use of fluorescent and light-emitting reporters to monitor virulence gene expression and protein localization

requires accurate visualization *in vivo*. This has been facilitated by significant enhancements of microscopic and flow cytometric technologies, detailed in § 2(c)(ii).

(i) *Microscopic approaches*

Image capture and digital analysis

The capture of images from fluorescent microscopes originally involved relatively insensitive video grabbing devices. CCD cameras offer significant improvements because they are composed of arrays of light-sensitive units (pixels) that allow imaging at higher resolution (Cinelli 1998; Entwistle 1998; Fung & Theriot 1998). This powerful tool can accurately detect weak signals associated with bacteria (Hinnebusch & Bendich 1997; Lewis *et al*. 1994). Improvement of image quality requires reliable background subtraction to improve contrast. To monitor bacterial gene expression *in vivo*, accurate quantification of signals is required. The development of digital imaging analysis has resolved this problem, allowing improved quality of both two-dimensional (2D) and three-dimensional (3D) images, and simplifying data storage (Shotton 1998).

Confocal microscopy

Confocal microscopy is an important development of light microscopy, which allows 3D analysis of relatively thick tissue sections. The confocal optical system resembles a moveable pinhole that admits light from a single focal plane only (Sheppard & Shotton 1997). This technique allows detection of fluorescing signal throughout tissue sections and is helpful for identifying the cell type in which bacteria reside. The localization of *S. typhimurium* in liver sections of mice was elegantly shown by confocal microscopy of immunohistochemical-stained sections (Richter-Dahlfors *et al*. 1997).

Image deconvolution

This software-based approach assembles 3D images by deblurring a library of images acquired with a conventional microscope to restore out-of-focus photons to their focus plane (Shaw 1995). The improved image contrast allows localization of the proteins in bacterial cells, and produces images that rival those obtained with a confocal microscope (Glaser *et al*. 1997).

Multiphoton microscopy

Confocal laser scanning microscopy has been limited by the lack of reliable fluorophores that could be excited by the ultraviolet part of the spectrum. One alternative is the two-photon-based molecular excitation system in which the sample is submitted to a stream of pulsed infrared light with a pulse frequency that makes one dye molecule likely to simultaneously absorb two long-photons in the focal plane. By absorbing two photons, the dye molecule combines their energy to reach its excited state and to fluoresce (Denk *et al*. 1990). This two-photon excitation can be used in conjunction with confocal microscopy to provide improved resolution of 3D images. Photobleaching, a problem with conventional fluorescent microscopy, is confined to the vicinity of the focal plane. As two-photon excitation allows sharp localization of fluorescence emission, this technique is useful for the observation of intracellular protein trafficking.

Microscopic analyses of reporter gene expression *in vivo* have several significant limitations: (i) high quality instruments (microscopes and CCD cameras) remain relatively expensive; (ii) animal tissues often give intense auto-fluorescence that overlays and masks weaker signals of interest; (iii) immunohistochemical techniques often require permeabilization of the specimen to allow reliable detection of epitopes; certain permeabilization solvents alter the fluorescence of reporter proteins such as GFP; (iv) deconvolution software is extremely processor intensive, making image processing lengthy.

Consequently, microscopic approaches are ideal for detecting bacteria and for determining the mammalian cell-type specificity of infecting bacteria. However, microscopy of tissue sections does not give a reliable measurement of bacterial gene expression but is limited to determining whether a gene is simply 'on' or 'off'. Accurate analyses of the expression of bacterial reporter genes *in vivo* require the use of flow cytometry and fluorescence-activated cell sorting (FACS).

(ii) *Flow cytometry*

Flow cytometry has been used extensively to study eukaryotic cells (Simons 1999) and its use for prokaryotic gene expression measurements is developing; it is a powerful technique for the assessment of intracellular fluorescence in individual bacterial cells. In combination with reporter gene technology, FACS can be used to analyse large populations of cells and to fractionate bacteria according to their level of gene expression. During FACS analysis, diffraction of argon laser light by bacteria gives information relating to size. This 488 nm light excites certain fluorophores causing emission of bacterial fluorescence which is amplified and detected. Signals are collected, digitized and stored for further analysis. Flow cytometry can measure the dynamics of gene induction in individual cells in response to environmental stimuli as discussed in § 2(c)(ii). Flow cytometric analysis of some bacterial species is complicated by aggregation, which can cause artefacts; *Mycobacteria* spp. require detergent and sonication treatment prior to cell sorting (Kremer *et al*. 1995; L. Ramakrishnan, R. Valdivia and S. Falkow, personal communication).

(c) *Techniques for identification of* in vivo*-induced genes*

Reporter genes have been used as the basis for selection procedures designed to isolate *in vivo*-induced (*ivi*) genes as outlined below. Several reviews have recently considered these techniques (Amann & Kühl 1998; Camilli 1996; Falkow 1997; Handfield & Levesque 1999; Relman & Wright 1998; Valdivia & Falkow 1997*b*, 1998). Analysis of *ivi* genes has already elucidated aspects of *in vivo* regulation of virulence gene expression and pathogen adaptation (Cotter & Miller 1998; Foster 1999; Guiney 1997; Mahan *et al*. 1996; VanBogelen *et al*. 1999).

(i) In vivo *expression technology*

The genetic approach of *in vivo* expression technology (IVET) allows the identification of host-induced genes during infection (Chiang *et al*. 1999; Handfield & Levesque 1999; Mahan *et al*. 1993). IVET relies on the insertion of random chromosomal fragments upstream of

a selectable gene that is required for survival in an animal model. The only bacteria that survive passage through the host must carry a fusion that expresses an active promoter. An *in vitro* pre-screening procedure ensures that only true *ivi* genes are identified.

IVET has been used to identify hundreds of *ivi* genes (Merrell & Camilli, this issue; Chiang *et al.* 1999; Handfield *et al.* 1998; Heithoff *et al.* 1997). The role of these genes in virulence is now being studied, and is complicated by the fact that between 25 and 50% of *ivi* genes do not resemble any other sequences in the database making it difficult to guess at their role; they are designated as having no known function (FUN) (Handfield & Levesque 1999; Hinton 1997). Auxotrophic and antibiotic resistance-based IVET selections are rather stringent and tend to identify genes that are highly expressed *in vivo*. These selection systems could also miss genes that are transiently expressed during infection. However, an improved recombinase fusion approach has been used to adapt IVET for the identification of genes expressed transiently during infection, which should allow the identification of organ-specific *ivi* genes (Camilli *et al.* 1994; Camilli & Mekalanos 1995; Merrell & Camilli, this issue).

(ii) *Differential fluorescence induction*

Differential fluorescence induction (DFI) is an alternative method for the positive selection of *ivi* genes during infection of cultured cells (Valdivia & Falkow 1997*a*). This system uses optimized GFP mutant proteins, which express up to 35-fold greater fluorescence than wild-type GFP and were selected by FACS (Cormack *et al.* 1996). These 'enhanced GFPs' (EGFP) allow individual bacteria bearing inducible gene fusions to be isolated by cell sorting (Valdivia *et al.* 1998). DFI involves the insertion of random genomic DNA upstream of the *gfp* reporter gene on a plasmid, followed by introduction to the bacterial pathogen. Bacteria harbouring *gfp* fusions are pooled and used for infection of cultured mammalian cells. FACS is used to isolate GFP-expressing bacteria released from lysed cells. These bacteria are cultivated on laboratory medium and a second FACS step is used to isolate bacteria with low *in vitro* fluorescence carrying *ivi* fusions that were only induced *in vivo*. Subsequent cell infection and bacteria sorting is used to enrich the *in vivo*-induced population. DFI was first applied to *S. typhimurium* to identify genes induced under acid conditions or within macrophages or epithelial cells (Valdivia *et al.* 1996; Valdivia & Falkow 1997*a*; Cirillo *et al.* 1998). A similar *gfp*-based approach has been used recently to identify macrophage-induced genes in *M. marinum* (Barker *et al.* 1998). In *M. smegmatis* and BCG, *gfp* transcriptional fusions to one heat-shock promoter *hsp60* have successfully been detected in animal tissue or infected macrophages using FACS, fluorescence and laser confocal microscopy (Dhandayuthapani *et al.* 1995; Kremer *et al.* 1995). DFI offers advantages to IVET for the identification of promoters that are only weakly and transiently induced during infection and offers accurate relative quantification of the level of gene expression in individual bacteria.

The analysis of bacterial GFP gene expression with flow cytometry does present some problems. Because sample exposure time is in the order of milliseconds in flow cytometric analysis, promoter strength is best detected and quantified by driving *gfp* expression from multicopy plasmids (Valdivia & Falkow 1997*a*). These limitations of detection explain why the current published examples describe plasmid-borne GFP reporter systems. Single-copy GFP expression has not been intense enough for accurate measurement. Our current reliance on plasmid-based GFP fusions prevents the detection of context- or topological-dependent effects of gene regulation.

(iii) *Limitations of* ivi *gene identification*

The IVET and DFI approaches identify *ivi* genes but do not address their role in virulence. The complementary approach of signature-tagged mutagenesis (STM) is a direct screen that allows the identification of *in vivo* survival or virulence-associated genes (Hensel *et al.* 1995). This technology is based on transposon mutagenesis and allows negative selection of genes whose expression is required for survival *in vivo* (Chiang & Mekalanos 1998; Hensel 1998; Unsworth & Holden, this issue).

The three approaches of IVET, DFI and STM can be used to recognize different classes of *ivi* or virulence-associated genes by testing the same gene library or mutant pool in different animal models. For example, STM has been used to screen for Tn*917* signature-tagged mutants of *Staphylococcus aureus* in the three models of mouse abscess, bacteraemia and wound infection (Coulter *et al.* 1998; Mei *et al.* 1997; Schwan *et al.* 1998).

IVET will not allow the identification of avirulence genes for which expression must be reduced *in vivo* to cause virulence (Galan 1998), but these could be recognized by a modified DFI approach.

The IVET and DFI *in vivo* expression technologies are based on transcriptional fusions and by definition will not detect genes that are post-transcriptionally regulated *in vivo*. Complementary approaches for identifying post-transcriptional induction *in vivo* are described below.

(d) *Direct measurement of bacterial mRNA levels in vivo*

The reporter-based systems described above have been used to follow mRNA expression indirectly. These systems are generally reliable, and much of the gene expression data obtained with reporter genes has subsequently been confirmed by direct monitoring of mRNA levels. However, reporter genes can occasionally give misleading data, which is only apparent when different systems for measuring gene expression are compared, as has been reported for *hns* and *proU* expression in *E. coli* and *Salmonella* (Forsberg *et al.* 1994; Free & Dorman 1995).

A number of techniques have been developed for the direct measurement of bacterial mRNA expression, as described below.

(i) *Subtractive hybridization*

A lack of genetic tools has prevented the application of reporter gene technology to many bacterial pathogens, and has led to the development of alternative techniques for the identification of *ivi* genes. Initially developed in eukaryotic cells, subtractive hybridization is now being used to study prokaryotic systems, such as the infection of cultivated macrophages by *M. avium* (Plum & Clark-Curtiss 1994). In

Table 1. *Approaches for monitoring bacterial gene expression* in vivo

techniques	organisms	cell type or animal	references
in vivo expression technology (IVET)	*Salmonella typhimurium*	mouse	Mahan *et al*. 1993
differential fluorescence induction (DFI)	*S. typhimurium*	epithelial cells, macrophages, dendritic cells	Valdivia & Falkow 1997
signature-tagged mutagenesis (STM)	*S. typhimurium*	mouse	Hensel *et al*. 1995
subtractive hybridization	*Mycobacterium avium*	macrophages	Plum & Clark-Curtiss 1994
differential display	uropathogenic *E. coli*	red blood cells	Zhang & Normark 1996
proteomic analysis	*S. typhimurium*	epithelial cells, macrophages	Buchmeier & Heffron 1990; Abshire & Neidhardt 1993
DNA microarrays	*Streptococcus pneumomiae* *Escherichia coli* *Mycobacterium tuberculosis*	none	De Saizeu *et al*. 1998; Richmond *et al*. 1999; Tao *et al*. 1999
in situ PCR	*S. typhimurium*	none	Tolker-Nielsen *et al*. 1997

this study, mycobacterial mRNA was converted to cDNA by reverse transcription. Biotin-labelled cDNA prepared from *M. avium* grown in broth was used to subtract constitutively expressed housekeeping genes from the cDNA of macrophage-derived *M. avium* using streptavidin-coated paramagnetic beads.

Unfortunately, the construction of representative cDNA libraries for subtractive hybridization is hampered by the instability of bacterial mRNA and the problems of isolating sufficient high-quality mRNA from small populations of bacteria growing *in vivo*. In addition, transiently expressed genes may not be well represented in the cDNA library. A PCR-based modification may prove to be a crucial innovation for subtractive hybridization, as it allows representation of low-abundance mRNA and requires a smaller number of bacteria (Sharma *et al*. 1993).

As well as being used to analyse gene expression, subtractive hybridization has also been applied to the study of genetic acquisition and bacterial evolution. Genomic subtractive hybridization was used for identifying virulence genes from strains of *Pseudomonas aeruginosa* isolated from cystic fibrosis patients (Schmidt *et al*. 1998) and genes that were specific to *S. typhimurium* and absent from *S. typhi* (Emmerth *et al*. 1999).

(ii) *Differential display*

Differential display was developed to identify eukaryotic genes that are induced under particular conditions (Kozian & Kirschbaum 1999), and is beginning to be applied to the identification of bacterial *ivi* genes.

This 'black-box' approach involves the construction of cDNA libraries from the mRNA of bacteria grown under different conditions. It consists of six steps: (i) isolation of RNA; (ii) reverse transcription; (iii) PCR amplification of cDNA species; (iv) electrophoretic separation of the resulting fragments; (v) reamplification of the fragments that vary between conditions, cloning and sequencing; and (vi) confirmation of the differential gene expression (Handfield & Levesque 1999).

Differential display has been used to identify virulence genes induced by adherence to red blood cells of uropathogenic *E. coli* to host cells (Zhang & Normark 1996) and to identify the novel *eml* locus in *Legionella pneumophila*, which is induced during early stages of macrophage infection (Abu Kwaik & Pederson 1996).

The differential display approach has the advantage of being applicable to more than two conditions at the same time, lending itself to multiplex analyses. Unlike the subtractive hybridization technique, differential display can detect both up- and downregulation of *in vivo*-induced genes. However, the combination of PCR with differential display commonly generates false-positive results that do not relate to *ivi* genes.

(e) *Proteomic approaches and* in vivo *expression*

The patterns of gene expression and the changes in mRNA levels shown by the above techniques simply reflect regulation at the transcriptional level which cannot be directly related to protein expression. Reporters such as alkaline phosphatase have been used to generate translational gene fusions to address this issue (Manoil & Beckwith 1985). However, the stability of such fusion proteins can be aberrant, giving misleading data concerning protein expression. To determine the role of post-transcriptional, translational or post-translational regulation *in vivo*, the entire bacterial protein complement (proteome) must be studied during infection. Approaches for proteome analysis are constantly being refined and improved, and are currently based on the 2D polyacrylamide gel electrophoresis technique (O'Farrell 1975). Separation from *in vitro*- and *in vivo*-grown bacteria, first by their isoelectric point and second by their molecular weight, allows detection of IVI proteins. Recent advances in mass spectrometry combined with increasing availability of genomic sequence data should allow IVI proteins to be identified for many pathogens (Humphery-Smith *et al*. 1997; O'Connor *et al*. 1998).

Two-dimensional gel electrophoresis has been used to characterize acid- and alkali-induced proteins during aerobic and anaerobic growth of *E. coli* (Blankenhorn *et al*. 1999) as well as *Salmonella* IVI proteins induced within host macrophages (Abshire & Neidhardt 1993; Buchmeier & Heffron 1990). IVI proteins of *S. typhimurium* that are expressed within Henle-407 cells were identified by using a labelled lysine precursor, which cannot be used by mammalian cells (Burns-Keliher *et al*. 1997). Two-dimensional gels showed that *S. typhimurium* exhibits

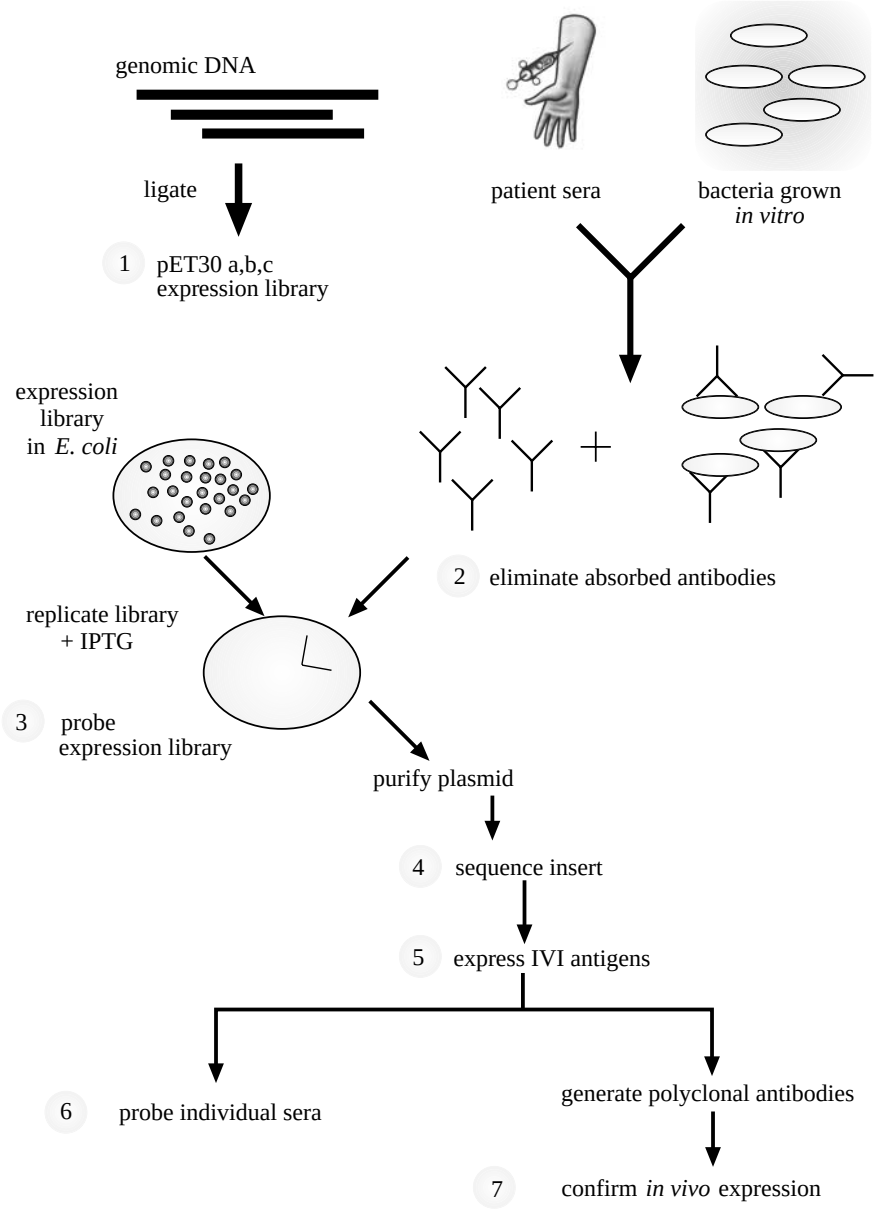

Figure 1. Diagram showing the principle of IVIAT (M. Handfield, personal communication). IPTG, isopropylthio-beta-D-galactoside.

cell-type- and species-specific gene expression in cultured cells (Burns-Keliher *et al*. 1998). However, this approach is limited because only a restricted set of proteins can be visualized on 2D gels. Furthermore, this technology has only been used in cultured cells and is unlikely to prove applicable to whole animal models. Unfortunately, the proteomic approach cannot assess protein expression in individual bacterial cells because of the quantities of protein currently required for 2D gel analysis.

3. FUTURE APPROACHES

The technologies outlined above are summarized in table 1, and have been used to identify hundreds of bacterial genes that are induced *in vivo*. Our current challenge is to determine the stage of pathogenesis at which these bacterial genes are expressed. Do *ivi* genes show organ-specific patterns of expression? Are *ivi* genes only switched on in certain cell types in the host? Are *ivi* genes switched on at early or late stages of infection? Do *ivi* genes show a temporal cascade of gene expression following induction? These questions require new approaches for monitoring virulence gene expression *in situ*.

(a) *Immunological-based detection of bacterial gene expression*

IVI virulence determinants can elicit a long-lived immune response in the host. Antibodies contained in sera from an infected host can subsequently be used to screen for bacterial IVI proteins. Comparative immunological detection of *Borrelia burgdorferi* proteins expressed *in vivo* or *in vitro* was achieved by screening a genomic expression library with sera obtained from infected or immunized mice after injection of live or killed pathogens

(Suk *et al*. 1995). These sera effectively discriminated between *in vivo*- and *in vitro*-expressed proteins.

Alternatively, IVI proteins can be recognized following immunization of individual mice with individual proteins encoded by each open reading frame (ORF) of a bacterial pathogen and screening for protection against subsequent challenge with wild-type bacteria. Sykes & Johnston (1999) described an elegant procedure involving the insertion of the promoterless ORF studied between a cytomegalovirus promoter and a eukaryotic terminator. These linear expression elements (LEE) generate transcriptionally active units, which were used individually to immunize mice. Specific immunization of the animals was checked on immunoblots with pathogen whole-cell lysates. This technique was used successfully to identify IVI proteins of *M. tuberculosis* (Felgner & Liang 1999). However, this complex strategy does require a complete genome sequence and careful optimization of the inoculum administration required to achieve a protective immune response.

An alternative method for monitoring protein expression involves the creation of protein fusions to specific epitope tags that can be identified immunologically. Induction of *spv* gene expression in *Salmonella* has been observed in murine macrophages by using transcriptional fusions of *spv* gene promoters and the fimbrial cluster of *E. coli* KS71A. Expression of *spv* genes was measured by agglutination of the bacteria with anti-fimbrial antibodies (Rhen *et al*. 1993).

(b) In vivo-*induced antigen technology*

A novel strategy to identify IVI proteins based on natural infection in humans has recently been described (M. Handfield, personal communication; figure 1). IVIAT was developed using sera from patients infected with *Actinobacillus actinomyce-temcomitans*, the aetiological agent of localized juvenile periodontitis, and offers several advantages to existing approaches. First and foremost, it is based on actual human infection and does not rely on potentially misleading animal models of disease. No particular genetic construct is required, allowing IVIAT to be used for any pathogen that can be grown *in vitro* (Handfield *et al*. 1999). This approach is rapid, simple and involves well-established methodologies, which facilitates the screening of a whole genome. Following growth of the pathogen *in vitro,* whole bacterial cells and total protein extracts are used to subtract antibodies contained in human patient sera that are directed against *in vitro*-expressed bacterial proteins. In parallel, a genomic expression plasmid library of the pathogen is constructed in *E. coli*. The remaining antibacterial antibodies in human sera are specific for IVI proteins and are subsequently used to identify these proteins from the expression library. A large amount of these specific antibodies can be purified and used to probe biological samples isolated from infected patients demonstrating *in vivo* protein induction via a direct immunofluorescence technique. IVIAT has been successfully used to study bacterial (*A. actinomyce-temcomitans* and *P. aeruginosa*) and yeast (*Candida* spp.) infections.

(c) In vivo *induction of fluorescence*

The limitations of luciferase and β-galactosidase reporter technology described earlier mean that a new approach was required for the monitoring of gene expression in individual bacterial cells *in vivo*. DFI relied on FACS to measure bacterial gene expression either *in vitro* or following isolation of bacteria from infected macrophages, epithelial cells or other mammalian cells. Our interest in organ-specific patterns of gene expression has led to the development of an improved system for assessing the transcriptional response of *Salmonella* virulence genes during murine infection (J. C. D. Hinton and I. Hautefort, unpublished data). *In vivo* induction of fluorescence (IVIF) uses a plasmid-based EGFP reporter system to monitor gene expression in tissues of infected mice. EGFP fluorescence can be detected and quantified in individual bacteria by fluorescent microscopic and flow cytometric analysis.

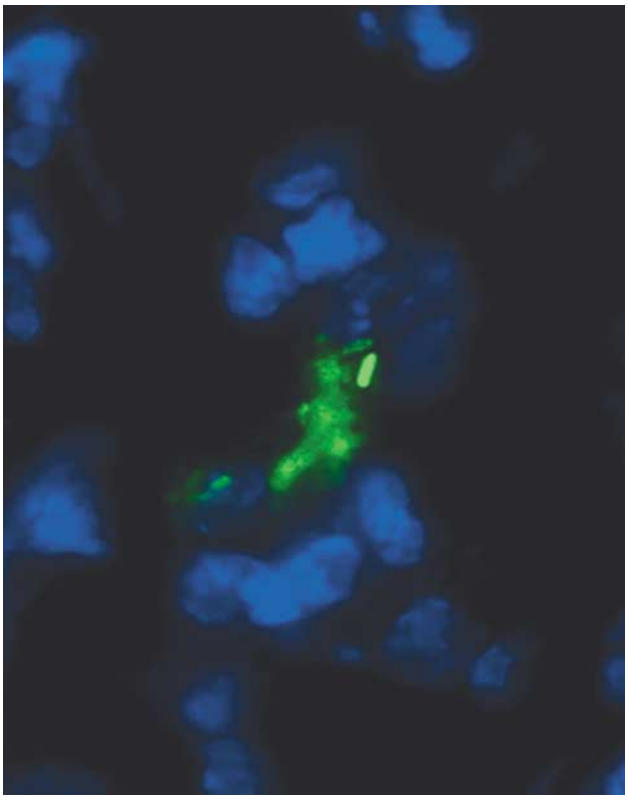

Figure 2. Fluorescence micrograph showing expression of a plasmid-borne transcriptional *ssrA:gfp* fusion in *S. typhimurium*. A DAPI-stained spleen section is shown from an intraperitoneally inoculated BALB/c mouse, with cell nuclei appearing as blue. Individual *S. typhimurium* 12023 bacteria expressing GFP appear as green (I. Hautefort, J. M. Sidebotham and J. C. D. Hinton, unpublished data).

The IVIF system does not perturb the invasion, spread or multiplication of *S. typhimurium* in mice. EGFP expression gives easily detectable fluorescence for both microscopic observations of organ sections and flow cytometric analysis on bacteria released from infected organs. The stability of EGFP (half-life >24 h) makes it ideal for the detection of transient gene induction *in vivo*.

Cultivated cell lines have already been used to determine gene induction kinetics (Valdivia & Falkow 1997*a*), but such cell-culture-based approaches cannot mimic the complex and changing environment that occurs at the site of infection. IVIF allows the identification of the pattern

of *ivi* gene expression by sacrificing mice at different time-points after infection and sampling from several sites. An example of the data that can be generated by IVIF (figure 2) shows the expression of a *S. typhimurium ssrA:gfp* fusion in an infected mouse spleen.

(d) In situ *PCR*

Although this technology has not yet been applied to the analysis of gene expression *in vivo*, a method for the measurement of intracellular mRNA levels is available. Hodson *et al.* (1995) outlined a method for *in situ* PCR of bacterial cells which was applied to microbial identification. More recently, Tolker-Nielsen *et al.* (1997) used *in situ* PCR on *Salmonella* to detect as little as one *lac* mRNA molecule per bacterial cell. The same method was used to demonstrate that *groEL* is cell-cycle regulated and that *lac* expression in *Salmonella* exhibited two distinct subpopulations (Holmstrom *et al.* 1999; Tolker-Nielsen *et al.* 1998). This *in situ* PCR approach has great promise for assessing gene expression in bacteria grown *in vitro*, and could potentially be applied *in vivo* to visualize organ-specific or cell-type-specific gene expression.

(e) *DNA microarrays*

A great many bacterial genome sequences are either now available or will be completed soon. These represent bacteria of academic, medical or industrial interest. Virulent *E. coli* and *Salmonella* genome sequences will shortly be released and the genomes of pathogenic organisms such as *Campylobacter* and *Neisseria* are already being exploited. The new 'functional genomic' approach involves concerted efforts to define gene function through analysis of global gene expression (transcriptome), protein expression (proteome) and genetics (construction of knockout strains). As an example of what is expected to be achieved in bacterial systems, DNA microarray analysis of the yeast genome has already identified hundreds of genes regulated during sporulation and yielded thousands of knockout strains with well-characterized phenotypes (Chu *et al.* 1998; Winzeler *et al.* 1999).

The availability of genome sequences has facilitated an important development of nucleic acid hybridization, the DNA microarray or chip. Microarrays are generally manufactured on glass microscope slides, and consist of an ordered grid of thousands of DNA spots. These can either be oligonucleotides or PCR products corresponding to every predicted ORF on the genome. Hybridization of labelled mRNA to the microarray allows the relative level of expression of each gene to be measured. This represents a significant breakthrough as it is now possible to get information for the expression of all bacterial genes from a single experiment.

The first bacterial application was described by De Saizeu *et al.* (1998) using an oligonucleotide-based microarray to look at expression of a subset of 100 genes from *Streptococcus pneumoniae*. Richmond *et al.* (1999) compared the reliability of DNA arrays by comparing arraying and labelling methods for *E. coli*. They concluded that DNA microarrays hybridized with fluorescently labelled samples gave the most reliable and sensitive data, and showed that this technology can be effectively applied to bacterial gene regulation. Tao *et al.* (1999) used an *E. coli* array to identify genes induced by growth on minimal media, and to recognize a new role for *rpoS* expression in exponentially growing cells.

DNA microarrays have the potential to revolutionize the study of *ivi* gene expression since they can yield global information concerning temporal and spatial aspects of gene expression. However, the limiting stage of microarray technology for *ivi* gene expression is the isolation of sufficient high-quality bacterial mRNA from infected tissue. Improved mRNA purification coupled with linear RNA amplification technology (Eberwine *et al.* 1992) could make microarrays the method of choice for *ivi* gene expression analysis in the future.

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