***In vivo* gene expression and the adaptive response: from pathogenesis to vaccines and antimicrobials**

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Microbial pathogens possess a repertoire of virulence determinants that each make unique contributions to fitness during infection. Analysis of these *in vivo*-expressed functions reveals the biology of the infection process, encompassing the bacterial infection strategies and the host ecological and environmental retaliatory strategies designed to combat them (e.g. thermal, osmotic, oxygen, nutrient and acid stress). Many of the bacterial virulence functions that contribute to a successful infection are normally only expressed during infection. A genetic approach was used to isolate mutants that ectopically expressed many of these functions in a laboratory setting. Lack of DNA adenine methylase (Dam) in *Salmonella typhimurium* abolishes the preferential expression of many bacterial virulence genes in host tissues. Dam[−] *Salmonella* were proficient in colonization of mucosal sites but were defective in colonization of deeper tissue sites. Additionally, Dam[−] mutants were totally avirulent and effective as live vaccines against murine typhoid fever. Since *dam* is highly conserved in many pathogenic bacteria that cause significant morbidity and mortality worldwide, Dams are potentially excellent targets for both vaccines and antimicrobials.

Keywords: vaccines; immunity; DNA adenine methylase; pathogenesis; *Salmonella*

1. INTRODUCTION

Similar to that of all microbes, a pathogen's primary objective is simply to survive and replicate (Falkow 1997). Key attributes that distinguish microbial pathogens from other micro-organisms include their ability to gain access to, replicate within, and persist at host sites that are forbidden to commensal species (Falkow 1997; Heithoff *et al.* 1997b). Such interactions within the host are often associated with pathological lesions; overt symptoms and disease are a consequence of a virulent organism achieving its goals using all evolutionary adaptations at its disposal. Thus, it can be argued that any function that contributes to the fitness of a pathogen within a host, or to its transmission to new hosts, may be viewed as a virulence factor. Since pathogenesis is a multifactorial process that is not restricted to a single pathway from infection to mortality, important virulence functions are often missed in standard virulence assays that require overt host injury (e.g. LD₅₀). Such virulence functions include those that are redundant, depend on the nutritional status of the host, depend on the presence of other host micro-organisms, and/or are involved in the transmission of the microbe to a new host. Thus, although the loss of some virulence functions may not cause a measurable change of host injury, they may make important contributions to the fitness of an organism within its natural host.

2. THE ADAPTIVE RESPONSE AND COORDINATE CONTROL OF GLOBAL VIRULENCE REGULONS

A more expansive definition of virulence functions includes factors involved in the adaptive response to environmental stresses since pathogens are exposed to alterations in temperature, osmolarity, oxygen tension, pH and nutrient availability during infection (Mahan *et al.* 1996; Mekalanos 1992; Miller *et al.* 1989). Indeed, several classical virulence functions are coordinately expressed with other genes in response to well-characterized environmental stresses duplicated in the laboratory. Consequently, such coordinately regulated genes can be considered members of global virulence regulons even if each individual gene does not make a direct contribution to pathogenesis. Moreover, independent of the actual (often unknown) *in vivo* signals, classification of bacterial genes based on regulatory patterns in a laboratory setting may reflect coordinate expression at a given anatomical site as well as a possible functional relationship within the host (e.g. survival within the small intestine or macrophage phagosome). For example *in vitro*, production of cholera toxin is coordinately regulated by the same environmental and genetic signals (e.g. pH, temperature, osmolarity, transcriptional activator ToxR) as the toxin co-regulated pilus (*tcp*); both of these virulence factors are required for the colonization and/or infection of the small intestine (Mahan *et al.* 1996; Waldor & Mekalanos 1996).

Additionally, environmental conditions can be established in a laboratory setting that appear to mimic micro-environments encountered within the host. Virulence

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genes that respond to these signals often belong to well-characterized global regulatory networks, and the changes that cells undergo in response to these environmental signals may themselves directly contribute to virulence. For example, low pH and low Mg^{2+} have been associated with *Salmonella*-containing vacuoles (Garcia-del Portillo *et al.* 1992) and have been shown to be relevant signals for bacterial genes presumed to function within the macrophage (Garcia Vescovi *et al.* 1996; Heithoff *et al.* 1999a; Soncini & Groisman 1996; Soncini *et al.* 1996). Thus, classification of virulence genes based on their coordinate response to environmental signals provides a means to understand the pathogenesis of the infecting microbe, the ecology of host-pathogen interactions, and the spatial and functional relationship between bacterial gene products and the cognate anatomical host sites at which they operate during infection. Moreover, investigation of gene expression patterns reveals the inherent versatility of pathogens to modify the expression of a given gene(s) at various host sites or within the context of different animal hosts.

Investigation into global adaptive responses controlling virulence gene expression may reveal novel regulatory mechanisms, virulence functions and mechanisms of transmission that each uniquely contribute to the fitness of a pathogen. For example, the ability to reduce the production of classical virulence functions such as adhesins, invasins and toxins may be as critical to bacterial survival as is the ability to produce them. This downregulation may be important to delay immune recognition, exercise economy and facilitate transmission to a new host. Additionally, downregulation of classical virulence functions in response to host environmental cues that favour bacterial growth (availability of nutrients, exposure to optimal temperature, oxygen tension and osmolarity) may be highly significant for bacterial fitness. Such a strategy may result in a subclinical infection that significantly enhances bacterial load while avoiding overt host injury. In time the disease path, however, would elicit immune recognition and other associated host response mechanisms that inhibit bacterial growth, which may ultimately force bacteria to take up residence at another anatomical site or within another host. Information gleaned from defining bacterial evolutionary adaptations, coupled with probing bacterial genes expressed within the host, may provide a means to understand further these microbial-host interactions.

3. METHODS TO INVESTIGATE *IN VIVO* GENE EXPRESSION

Analysis of bacterial-host interactions is complicated by the fact that not all virulence factors confer a selective advantage at the same stage of infection or at the same anatomical site within the host. Accordingly, expression of certain virulence factors must be modulated in response to signals determining the transition from external environments to growth within the natural host and to other environmental signals encountered throughout the infection process. Moreover, individual responses may not all exist in the same bacterial cell or, alternatively, the overall response may be the additive result of different responses occurring in different cells. Thus, mimicking

these environments in the laboratory is problematic since the micro-environments within host tissues are both complex and dynamic; gene expression is a consequence of all of these contributions.

Many of these limitations have been overcome, in part, by recent *in vivo* expression methods that allow the isolation of bacterial genes that are expressed during infection. There are many fundamentally different methods for identifying *in vivo*-expressed genes (Heithoff *et al.* 1997b). A survey of *in vivo* expression methods reveals that the most effective approach is to use them in combination, since they each contribute uniquely to our understanding of pathogenesis, virulence and infection.

(a) *In vivo* expression technology

In vivo expression technology (IVET) is a promoter trap wherein bacterial promoters drive the expression of a gene that is required for virulence; complementation in the animal demands elevated levels of expression compared with growth on a laboratory medium (Mahan *et al.* 1993, 1995). The advantage is that the β -galactosidase reporter system can be used to explore functional, spatial and regulatory relationships between genes normally expressed during infection. The disadvantage is that mutants in desired genes must be constructed individually and subsequently tested for virulence.

(b) Signature-tagged mutagenesis

Signature-tagged mutagenesis (STM) is a negative selection strategy in which a pool of tagged insertion mutants is used to inoculate an animal; mutations represented in the initial inoculum but not recovered from an infected animal are required for infection (Hensel *et al.* 1995). The advantage is that individual sequence tags serve as insertion mutations that can be tested in virulence assays. The disadvantage is that the mutations are genetically intractable for further analysis.

(c) Differential display

Differential display (DD) is a subtractive hybridization strategy in which bacterial cDNAs from infected tissue are hybridized against a cDNA library constructed from laboratory-grown bacteria (Abu Kwaik & Pederson 1996; Plum & Clarke-Curtiss 1994); the resulting host-specific cDNAs are used as probes to isolate *in vivo*-expressed bacterial genes. The advantage is that DD is applicable to many important pathogens lacking defined genetic systems. The disadvantage is that mutants in desired genes must be constructed individually and subsequently tested for virulence.

(d) Known virulence genes

Table 1 shows that many known virulence genes have been identified using *in vivo* expression methods in a wide variety of host-pathogen systems. Examples include adhesins and colonization factors making up type III secretion systems, which are involved in host-contact-dependent secretion of virulence functions; *tcp*, required for *Vibrio cholerae* colonization of the mouse small intestine; and B2, a *Proteus mirabilis* virulence factor required for bladder colonization in a murine model of urinary tract infection. Other virulence functions are involved in intracellular and/or systemic survival, including *spvB*, a *Salmonella*

Table 1. *Microbial genes expressed during infection*

(Listed are functions or attributes of *in vivo*-expressed genes, their known or inferred role in pathogenesis, the host tissue or condition in which they were selected, and the methodology and organism from which they were recovered. DFI (differential fluorescence induction) is an IVET-like selection strategy involving the green fluorescent protein reporter.)

gene	function	role in pathogenesis	tissue	method	species	references
adherence, colonization, invasion and secretion						
SPI-2	type III secretion system	invasion and systemic survival	mouse spleen, macrophage line	STM, DFI	<i>Salmonella typhimurium</i>	Hensel <i>et al.</i> (1995), Shea <i>et al.</i> (1996), Valdivia & Falkow (1997)
<i>yscCLRU</i> , <i>virG</i> , <i>lcrV</i>	type III secretion system	systemic survival	mouse spleen	STM	<i>Yersinia enterocolitica</i>	Darwin & Miller (1999)
<i>bscN</i>	type III secretion system	colonization	rat trachea	DD	<i>Bordetella bronchiseptica</i>	Yuk <i>et al.</i> (1998)
<i>rhi-18</i>	type III secretion system	colonization	sugar-beet roots	IVET	<i>Pseudomonas fluorescens</i>	Rainey (1999)
<i>tcpAEFT</i>	toxin co-regulated pilus	adhesion and colonization	mouse intestine	STM	<i>Vibrio cholerae</i>	Chiang & Mekalanos (1998)
<i>EPA1</i>	adherence	adhesion	epithelial cell line	STM	<i>Candida glabrata</i>	Cormack <i>et al.</i> (1999)
B2	protease/collagenase	colonization	mouse bladder	STM	<i>Proteus mirabilis</i>	Zhao <i>et al.</i> (1999)
<i>iviVI-A</i> , <i>iviVI-B</i>	Tia-like, PfEMP1-like	adhesion and invasion	mouse spleen	IVET	<i>Salmonella typhimurium</i>	Heithoff <i>et al.</i> (1997a)
<i>ivi131-19</i>	Hag2-like	adhesion and invasion	rat lung, mouse liver	IVET	<i>Pseudomonas aeruginosa</i>	Handfield <i>et al.</i> (2000)
cutinase-like gene	cutinase	invasion	pepper roots	DD	<i>Phytophthora capsici</i>	Munoz & Bailey (1998)
<i>secE</i>	secretion	protein export	pig lung	IVET	<i>Actinobacillus pleuropneumoniae</i>	Fuller <i>et al.</i> (1999)
intracellular and systemic survival						
spvRAD, <i>spvB</i>	plasmid virulence	systemic survival	mouse spleen	STM, IVET	<i>Salmonella typhimurium</i>	Hensel <i>et al.</i> (1995), Heithoff <i>et al.</i> (1997a)
<i>eml</i>	early macrophage infection	macrophage survival	macrophage line	DD	<i>Legionella pneumophila</i>	Abu Kwaik & Pederson (1996)
SAP2	secreted aspartic proteases	disseminated candidiasis	mouse spleen	IVET	<i>Candida albicans</i>	Staib <i>et al.</i> (1999)
<i>dotB</i>	organelle trafficking	macrophage survival	guinea-pig lung and spleen	STM	<i>Legionella pneumophila</i>	Edelstein <i>et al.</i> (1999)
<i>icmX</i>	intracellular multiplication	macrophage survival	guinea-pig lung and spleen	STM	<i>Legionella pneumophila</i>	Edelstein <i>et al.</i> (1999)
<i>mig</i>	unknown	macrophage survival	macrophage line	DD	<i>Mycobacterium avium</i>	Plum <i>et al.</i> (1997)
Rv0288	ESAT.6	Tcell antigen	unknown	DD	<i>Mycobacterium tuberculosis</i>	Rindi <i>et al.</i> (1999)
Rv2770c	PPE protein family	antigenic variation and	unknown	DD	<i>Mycobacterium tuberculosis</i>	Rindi <i>et al.</i> (1999)
MTV041.29	PPE protein family	inhibit antigen processing	unknown	DD	<i>Mycobacterium tuberculosis</i>	Rivera-Marrero <i>et al.</i> (1998)
Rv1345	polyketide synthase	lipid and/or metabolite synthesis	unknown	DD	<i>Mycobacterium tuberculosis</i>	Rindi <i>et al.</i> (1999)
nutrient acquisition and synthesis						
metals						
<i>entF</i>	siderophore	Fe ²⁺ uptake	macrophage line	IVET	<i>Salmonella typhimurium</i>	Heithoff <i>et al.</i> (1997a)
<i>irp1</i>	siderophore	Fe ²⁺ uptake	mouse spleen	STM	<i>Yersinia enterocolitica</i>	Darwin & Miller (1999)
<i>fhuA</i> , <i>cirA</i>	iron transport	Fe ²⁺ uptake	mouse intestine, macrophage line	IVET	<i>Salmonella typhimurium</i>	Heithoff <i>et al.</i> (1997a)
<i>fyuA</i>	iron transport	Fe ²⁺ uptake	mouse Peyer's patch	IVET	<i>Yersinia enterocolitica</i>	Young & Miller (1997)
<i>fptA</i>	iron transport	Fe ²⁺ uptake	mouse liver	IVET	<i>Pseudomonas aeruginosa</i>	Wang <i>et al.</i> (1996)

(Cont.)

Table 1 (Cont.)

gene	function	role in pathogenesis	tissue	method	species	references
<i>pydD</i>	pyoverdin synthesis	Fe ²⁺ acquisition	rat lung and mouse liver	IVET	<i>Pseudomonas aeruginosa</i>	Handfield <i>et al.</i> (2000)
<i>np20</i>	Fur-like	Fe ²⁺ starvation response	mouse liver	IVET	<i>Pseudomonas aeruginosa</i>	Wang <i>et al.</i> (1996)
<i>mgtA, mgtB</i>	Mg ²⁺ transport	Mg ²⁺ uptake	mouse spleen, macrophage line	IVET	<i>Salmonella typhimurium</i>	Heithoff <i>et al.</i> (1997a)
<i>mgtE</i>	Mg ²⁺ transport	Mg ²⁺ uptake	mouse intestine	STM	<i>Vibrio cholerae</i>	Chiang & Mekalanos (1998)
<i>iviX</i>	Cu ²⁺ transport	Cu ²⁺ homeostasis	macrophage line	IVET	<i>Salmonella typhimurium</i>	Handfield <i>et al.</i> (2000)
nucleotides						
<i>carAB, purDL</i>	pyrimidine, purine synthesis	<i>de novo</i> requirement	mouse spleen	IVET, STM	<i>Salmonella typhimurium</i>	Mahan <i>et al.</i> (1993), Hensel <i>et al.</i> (1995)
<i>purCELK</i>	purine synthesis	<i>de novo</i> requirement	mouse lung	STM	<i>Streptococcus pneumoniae</i>	Polissi <i>et al.</i> (1998)
<i>purDHK</i>	purine synthesis	<i>de novo</i> requirement	mouse intestine	STM	<i>Vibrio cholerae</i>	Chiang & Mekalanos (1998)
<i>purL</i>	purine synthesis	<i>de novo</i> requirement	mouse spleen	STM	<i>Staphylococcus aureus</i>	Mei <i>et al.</i> (1997)
<i>vacB, vacC</i>	mRNA, tRNA processing	post-transcriptional regulation	mouse spleen, mouse intestine	IVET	<i>Salmonella typhimurium</i>	Heithoff <i>et al.</i> (1997a)
<i>vacC</i>	tRNA processing	post-transcriptional regulation	mouse Peyer's patch	IVET	<i>Yersinia enterocolitica</i>	Young & Miller (1997)
cofactors						
<i>hemA</i>	haem synthesis	peroxide resistance	mouse spleen	IVET	<i>Salmonella typhimurium</i>	Heithoff <i>et al.</i> (1997a)
<i>hemD</i>	haem synthesis	peroxide resistance	mouse Peyer's patch	IVET	<i>Yersinia enterocolitica</i>	Young & Miller (1997)
<i>cobI</i>	vitamin B ₁₂ synthesis	carbon source use	mouse liver	IVET	<i>Pseudomonas aeruginosa</i>	Wang <i>et al.</i> (1996)
<i>iviXVII</i>	B ₁₂ synthesis, propanediol use	carbon source use	mouse spleen	IVET	<i>Salmonella typhimurium</i>	Heithoff <i>et al.</i> (1997a)
<i>bioB</i>	biotin synthetase	biotin synthesis	mouse intestine	STM	<i>Vibrio cholerae</i>	Chiang & Mekalanos (1998)
stress response						
DNA repair						
<i>recD</i>	recombination and repair	macrophage survival	macrophage line	IVET	<i>Salmonella typhimurium</i>	Heithoff <i>et al.</i> (1997a)
<i>recB</i>	recombination and repair	macrophage survival	mouse Peyer's patch	IVET	<i>Yersinia enterocolitica</i>	Young & Miller (1997)
<i>radA</i>	recombination and repair	macrophage survival	mouse lung	STM	<i>Streptococcus pneumoniae</i>	Polissi <i>et al.</i> (1998)
<i>recA</i>	recombination and repair	macrophage survival	mouse liver and spleen	STM	<i>Staphylococcus aureus</i>	Coulter <i>et al.</i> (1998)
<i>mutL</i>	DNA repair	intestine survival	mouse Peyer's patch	IVET	<i>Yersinia enterocolitica</i>	Young & Miller (1997)
<i>hexA</i>	DNA repair	systemic survival	mouse liver and spleen	STM	<i>Staphylococcus aureus</i>	Coulter <i>et al.</i> (1998)
environmental						
<i>ompR/envZ</i>	osmoregulation	osmotic protection	mouse spleen	STM	<i>Salmonella typhimurium</i>	Hensel <i>et al.</i> (1995)
<i>otsA</i>	trehalose synthesis	thermo/osmotic protection	mouse intestine	IVET	<i>Salmonella typhimurium</i>	Heithoff <i>et al.</i> (1997a)
<i>rhi-12</i>	IsfA-like	oxidative stress	sugar-beet roots	IVET	<i>Pseudomonas fluorescens</i>	Rainey <i>et al.</i> (1997)
<i>rhiI</i>	glycine–betaine binding	osmotic protection	sugar-beet roots	IVET	<i>Pseudomonas fluorescens</i>	Rainey <i>et al.</i> (1997)
<i>cadC</i>	cadaverine synthesis	acid tolerance	mouse intestine and spleen	IVET	<i>Salmonella typhimurium</i>	Heithoff <i>et al.</i> (1997a)
<i>cadA</i>	cadaverine synthesis	acid tolerance	rabbit intestine	IVET	<i>Vibrio cholerae</i>	Merrell & Camilli (2000)
<i>dnaJ</i>	protein chaperone	heat shock response	mouse spleen	STM	<i>Yersinia enterocolitica</i>	Darwin & Miller (1999)

(Cont.)

Table 1 (Cont.)

gene	function	role in pathogenesis	tissue	method	species	references
<i>fadB</i>	fatty acid degradation	clear pro-inflammatory	mouse spleen	IVET	<i>Salmonella typhimurium</i>	Mahan <i>et al.</i> (1995)
<i>hylC</i>	secreted lipase	fatty acids	mouse intestine	IVET	<i>Vibrio cholerae</i>	Camilli & Mekalanos (1995)
<i>lip</i>	lipid degradation		mouse kidney	IVET	<i>Staphylococcus aureus</i>	Lowe <i>et al.</i> (1998)
<i>cfa, aas</i>	membrane modifications	membrane repair	mouse intestine and spleen, macrophage cell	IVET, DFI	<i>Salmonella typhimurium</i>	Heithoff <i>et al.</i> (1997 <i>a</i>), Valdivia & Falkow (1996)
<i>ivi134–21</i>	membrane modifications	protein targeting	mouse liver and rat lung	IVET	<i>Pseudomonas aeruginosa</i>	Handfield <i>et al.</i> (2000)
chemotaxis <i>iviIV</i>	signal receptor	chemoreception	mouse intestine	IVET	<i>Vibrio cholerae</i>	Camilli & Mekalanos (1995)
<i>np9</i>	response regulator	chemoreception	mouse liver	IVET	<i>Pseudomonas aeruginosa</i>	Wang <i>et al.</i> (1996)
regulatory functions <i>phoP</i>	virulence regulator	invasion and macrophage survival	mouse spleen	IVET	<i>Salmonella typhimurium</i>	Heithoff <i>et al.</i> (1997 <i>a</i>)
<i>toxT</i>	transcription activator	colonization regulator	mouse intestine	STM	<i>Vibrio cholerae</i>	Chiang & Mekalanos (1998)
<i>pmrB</i>	polymyxin resistance	neutrophil survival	macrophage line	IVET	<i>Salmonella typhimurium</i>	Heithoff <i>et al.</i> (1997 <i>a</i>)
<i>pspC</i>	phage shock protein regulation	unknown	mouse spleen	STM	<i>Yersinia enterocolitica</i>	Darwin & Miller (1999)
<i>agrA</i>	accessory gene regulation	virulence gene regulation	mouse kidney	IVET	<i>Staphylococcus aureus</i>	Lowe <i>et al.</i> (1998)
<i>hre-7</i>	AcrR-like	efflux pump regulator	mouse Peyer's patch	IVET	<i>Yersinia enterocolitica</i>	Young & Miller (1997)
<i>rhi-3</i>	CopRS-like	copper-inducible regulator	sugar-beet roots	IVET	<i>Pseudomonas fluorescens</i>	Rainey (1999)
B5	nitrogen response regulation	colonization	mouse bladder	STM	<i>Proteus mirabilis</i>	Zhao <i>et al.</i> (1999)
membrane/cell wall <i>rfb</i>	lipopolysaccharide synthesis	cell integrity	mouse spleen	IVET, STM	<i>Salmonella typhimurium</i>	Mahan <i>et al.</i> (1993), Hensel <i>et al.</i> (1995)
<i>rfb</i>	lipopolysaccharide synthesis	cell integrity	mouse intestine	STM	<i>Vibrio cholerae</i>	Chiang & Mekalanos (1998)
<i>galE</i>	lipopolysaccharide synthesis	cell integrity	mouse spleen	STM	<i>Yersinia enterocolitica</i>	Darwin & Miller (1999)
<i>femA, femB</i>	peptidoglycan cross-linking	cell wall assembly	mouse spleen	STM	<i>Staphylococcus aureus</i>	Mei <i>et al.</i> (1997)
<i>pbp2</i>	peptidoglycan cross-linking	cell wall assembly	mouse kidney	IVET	<i>Staphylococcus aureus</i>	Lowe <i>et al.</i> (1998)
<i>pbp2</i>	penicillin-binding protein	cell wall maintenance	mouse kidney	IVET	<i>Staphylococcus aureus</i>	Lowe <i>et al.</i> (1998)
2.9-71pB	lipoprotein	unknown	rat peritoneum	DD	<i>Borrelia burgdorferi</i>	Akins <i>et al.</i> (1998)
<i>nlpD</i>	lipoprotein	unknown	mouse spleen	STM	<i>Yersinia enterocolitica</i>	Darwin & Miller (1999)

plasmid-encoded virulence function required for growth at systemic sites of infection; *eml*, a *Legionella pneumophila* virulence function involved in the early stages of macrophage infection; and SAP2, a *Candida albicans* virulence function involved in late-stage disseminated infection.

The most common classes of *in vivo*-expressed genes are those involved in nutrient acquisition and the stress response, suggesting that these genes contribute significantly to the fitness of the micro-organism during infection. Examples include the acquisition of metals such as Fe^{2+} , Mg^{2+} , Cu^{2+} , the acquisition and synthesis of nucleotides and co-factors, and the induction of genes involved in DNA repair and thermo-, osmotic and acid tolerance. These *in vivo*-expressed genes reveal important insights into host ecology, which plays a dual role in pathogenesis: to induce the activation of bacterial genes that complement nutrient-limiting conditions in the host and to induce the activation of other virulence genes required for immediate survival and spread to subsequent anatomical sites of infection.

Determination of the genetic and environmental factors that control virulence genes and the host site(s) in which they are expressed provides clues to both the intracellular environment and possible functions and/or functional relationships of these genes at these specific anatomical sites. For example, many IVET-selected bacterial strains recovered from infected spleens and/or from cultured macrophages were grouped based on their response to environmental and genetic signals that are believed to be involved in bacterial gene expression in the macrophage phagosome (low pH, low Mg^{2+}) (Mahan *et al.* 1996; Mekalanos 1992; Miller *et al.* 1989). Intracellular expression studies showed that each of the low pH and low Mg^{2+} responsive *in vivo*-induced (*ivi*) fusion strains is induced upon entry into and growth within three distinct mammalian cell lines, including murine macrophages and two cultured human epithelial cell lines (Heithoff *et al.* 1999a). This suggests that this class of coordinately expressed *ivi* genes responds to general intracellular signals that are present both in initial and in progressive stages of infection and may reflect their response to similar vacuolar micro-environments in these cell types. Determination of the genetic and environmental factors that regulate gene expression at their cognate host site(s) provides clues to both the intracellular environment and possible gene functions at these anatomical sites. Moreover, the functions of some of these genes may change dependent upon the context of the animal, tissue, cell type or subcellular compartment.

4. PILI EXPRESSION, DNA METHYLATION AND INTEGRATION WITH THE ADAPTIVE RESPONSE

Pili (fimbriae) are appendages that allow bacteria to adhere to host cells and to other bacteria. Environmental conditions controlling pili expression can be established in the laboratory setting that appear to mimic, at least in part, micro-environments encountered within the host (reviewed by Krabbe *et al.* 2000). The expression of many pili types is controlled by complex and overlapping regulatory circuitry involving a DNA methylation system that integrates pili expression with the adaptive response. Such regulation is best understood in uropathogenic *Escherichia*

coli wherein DNA adenine methylase (Dam) controls the expression of pyelonephritis-associated pili (Pap), which are essential for colonization of the urogenital tract. Genes encoding Pap are reversibly switched between the unexpressed state and the expressed state, termed phase variation, a process that is exquisitely sensitive to environmental signals such as temperature and carbon source and to a number of DNA-binding proteins. Pap phase variation is principally controlled by Dam, which binds to the sequence GATC in double-stranded DNA and methylates adenine at the *N*-6 position (Marinus 1996). Dam controls several important and diverse biological processes including DNA replication, methyl-directed mismatch repair (MDMR), transposition and the expression of pili required for colonization of host tissues (Marinus 1996; Van der Woude *et al.* 1996). The molecular basis of this versatility lies in its ability to chemically modify DNA (by methylation), which imparts additional information to the DNA primary sequence.

Although Dam methylates most of the approximately 18 000 GATC sites in the *E. coli* chromosome, certain GATC sites are protected from methylation by the binding of regulatory proteins at or near these sites, forming specific DNA methylation patterns similar to those observed in eukaryotes (Hale *et al.* 1994; Tavazoie & Church 1998). Studies on the *pap* operon have shown that DNA methylation patterns directly control the ON–OFF switch regulating Pap expression in uropathogenic *E. coli* (Braaten *et al.* 1992, 1994; Van der Woude *et al.* 1992, 1996; Van der Woude & Low 1994). Methylation of one of the GATC sites in the *pap* regulatory region inhibits the binding of the global regulator leucine responsive regulatory protein (Lrp), providing a mechanism by which Dam controls *pap* gene expression (Nou *et al.* 1993; Van der Woude *et al.* 1996). Both genetic analysis and DNA database searching for conserved GATC box motifs present in *pap* indicates that there are at least 16 additional pili operons in various *E. coli* and *Salmonella* strains that are probably under DNA methylation control (Krabbe *et al.* 2000; Van der Woude & Low 1994). In addition to regulating pili gene expression, Dam has also been reported to control the expression of the phase variable Agg43 outer membrane protein in *E. coli*. In this case it appears that methylation of a regulatory DNA GATC site inhibits the binding of the redox-sensing transcriptional activation OxyR, here acting as a repressor (Henderson *et al.* 1997), tying the DNA methylation state to the oxidative stress response.

Examination of pili regulatory mechanisms reveal that a series of DNA–protein and protein–protein interactions make up a regulatory network that integrates pili expression with adaptive response pathways. Various environmental stimuli control the expression of DNA-binding proteins such as Lrp. Lrp binds to specific sequences containing GATC sites, blocking their methylation and forming specific DNA methylation patterns (Braaten *et al.* 1994). These DNA methylation patterns may constitute a type of ‘bacterial memory’ since they are heritable to subsequent generations. Thus pili expression serves an important paradigm to understand the complex regulatory circuitry that integrates environmental signals with the *in vivo* response of bacterial pathogens.

5. COMBINING THE GLOBAL REGULATORY ADAPTIVE AND METHYLATION RESPONSE SYSTEMS WITH IVET TO REVEAL BACTERIAL VIRULENCE STRATEGIES

Combining global regulatory adaptive and methylation response systems with *in vivo* expression methods yields many powerful experimental tools to reveal bacterial infection strategies and the ensuing host responses. For example, conceptually, the IVET strategy selects for genes that are ON inside the mouse and OFF outside the mouse. Such coordinate expression in response to the transition from host tissues to the laboratory setting suggested a master genetic switch(es) controlling *ivi* gene expression. Accordingly, *lac* fusion technology inherent in the IVET approach was exploited to screen for regulatory mutants that exhibited the phenotype: ON inside the mouse and ON outside the mouse. It was anticipated that this class of mutants should not undergo differential expression in host tissues, and consequently, such mutants should be severely attenuated in their ability to cause disease.

(a) *Dam* is a global regulator of bacterial genes induced during infection

Mutations in *Dam* answered this genetic selection, repressing the expression of greater than 20% of over 100 *Salmonella* *ivi* genes (Heithoff *et al.* 1999b). Several of these *Dam*-regulated *ivi* genes have been shown to be involved, or have been implicated, in virulence, and are controlled by environmental signals in the laboratory that are presumed to reflect micro-environments within the host (e.g. low pH, low Mg^{2+} and/or low iron). These results indicate that *Dam* is a global regulator of *Salmonella* gene expression and that the *dam*-regulated *ivi* genes constitute a *dam* regulon as suggested by Marinus (1996).

(b) *Dam* is essential for pathogenesis

Since *Dam* is a master switch controlling genes that are specifically induced during infection, its effect on virulence was determined in a murine model of typhoid fever. *Dam* plays an essential role in *Salmonella* pathogenesis as strains that lack *Dam* contain a severe virulence defect (greater than 10 000-fold) in that they are unable to cause murine typhoid fever (Heithoff *et al.* 1999b). Mutational analysis indicates that the reduction in virulence of *Dam*⁻ *Salmonella* strains was not due to the loss of methyl-directed mismatch repair (MDMR), or *Lrp*, since *mutS* and *lrp* mutants are fully virulent. Thus, *Dam*'s global regulatory role in *Salmonella* virulence is clearly distinct from its role in MDMR and its role with *Lrp* controlling pili expression in *E. coli* (Braaten *et al.* 1994).

(c) *Dam*, *PhoP* and DNA methylation patterns

Salmonella pathogenesis is known to be controlled by *PhoP*, a DNA-binding protein that acts as both an inducer and repressor of specific virulence genes (reviewed by Groisman & Heffron 1995). Binding of regulatory proteins to DNA can form DNA methylation patterns by blocking methylation of specific *Dam* target sites (GATC sequences) (Van der Woude *et al.* 1998). Thus, it was determined whether binding of *PhoP* (or a *PhoP*-

regulated protein) to specific DNA sites blocks methylation of these sites by *Dam*, resulting in an alteration in the DNA methylation pattern (following *MboI* cleavage and pulsed-field gel analysis). *Salmonella* *PhoP*⁺ and *PhoP*⁻ DNA methylation patterns showed distinct differences, suggesting that binding of *PhoP* blocked methylation at specific GATC sites (Heithoff *et al.* 1999b). Moreover, *Salmonella* GATC sites protected from methylation are likely to be within gene regulatory regions since almost all GATC sites protected from methylation in *E. coli* are in 5' non-coding DNA regions that are presumably involved in the control of gene expression (Hale *et al.* 1994; Tavazoie & Church 1998). Methylation of specific GATC sites in gene regulatory regions could modulate the binding of regulatory proteins to DNA, which in turn provides a mechanism to control virulence gene expression.

(d) *Dam*⁻ mutants serve as live attenuated vaccines

Live attenuated vaccines contain living organisms that are benign but can replicate in host tissues and presumably express many natural immunogens similar to those generated during infection. This interaction elicits a protective response just as if the immunized individual had been previously exposed to the disease. Most of the work defining attenuating mutations for the construction of live bacterial vaccines has been done in *Salmonella* spp., since they establish an infection by direct interaction with the gut-associated lymphoid tissue, resulting in a strong humoral immune response. They also invade host cells and thus are capable of eliciting a strong cell-mediated response (Hassan & Curtiss 1997; Hormaeche *et al.* 1996; Miller *et al.* 1990).

Since *Dam* represses the expression of many *S. typhimurium* genes that are normally only expressed in animal hosts, in the absence of *Dam*, these genes and their cognate proteins should be ectopically expressed by bacteria when grown on laboratory media (Heithoff *et al.* 1999b). Consequently, *Dam*⁻ strains were tested for their protective capacity as live attenuated vaccines. Mice immunized with *Dam*⁻ mutants were completely protected against a wild-type challenge of 10 000-fold above the LD₅₀ of virulent *Salmonella* (Heithoff *et al.* 1999b), indicating that *Dam*⁻ mutants serve as live vaccines against murine typhoid fever. Additionally, *Dam*⁻ *Salmonella* were shown to survive in Peyer's patches of the mouse small intestine at wild-type levels, yet were unable to invade and/or survive in systemic tissues (Heithoff *et al.* 1999b). Moreover, it was recently shown that *Dam*⁻ bacteria are mildly deficient in the invasion of epithelial cells and the secretion of type III effector molecules and are less cytotoxic to M cells, yet they retain their protective capacity (Garcia-del Portillo *et al.* 1999). Taken together, these data indicate how the loss of a single enzyme can completely block the ability of a pathogen to cause disease yet elicit a fully protective immune response.

These studies offer two exciting opportunities for further research. (i) *Dams* are highly conserved in many pathogenic bacteria that cause significant morbidity and mortality worldwide including *V. cholerae* (cholera), *Shigella* spp. (dysentery), *Haemophilus influenzae* (meningitis; ear infection), *Salmonella typhi* (typhoid fever), and pathogenic *E. coli* and *Yersinia pestis* (plague) (Heithoff *et al.* 1999b). If

Dam works as a master switch in these organisms as it does in *Salmonella*, the construction of Dam-based live vaccines and antimicrobials that inhibit the Dam enzyme may result in a new generation of therapeutic agents to combat these infectious diseases. (ii) Since Dam is a global repressor of genes expressed during infection, Dam⁻ mutants of these organisms may inappropriately express many immunogens, which may elicit a cross-protective response against related pathogenic strains. Moreover, it may be possible to construct Dam-based vaccines in strains that are less harmful to humans, yet capable of conferring full protection against related pathogenic strains. Such vaccines would exploit the benefits of the high levels of protection elicited by live vaccines, while reducing the risk of infection to immunocompromised individuals.

6. CONCLUSIONS AND UNANSWERED QUESTIONS

The reactinogenicity (e.g. symptoms such as diarrhoea) of current live bacterial vaccine candidates and the reality that many individuals within the human population are immunocompromised clearly warrants the search for additional vaccines that offer better protection, are longer lasting, and have less toxicity. Use of *in vivo* expression methods in combination with the adaptive and/or methylation global regulatory response systems provides a powerful means to understand pathogenesis, virulence and infection. Such a multifaceted experimental strategy has broad application towards the design of a new generation of vaccines and antimicrobials to target a wide variety of infectious agents.

Important unanswered questions for future research include: (i) Are mutants lacking Dam attenuated in other pathogens, and if so, do they elicit a fully protective immune response? (ii) Will Dam⁻ vaccines constructed in less pathogenic strains elicit a cross-protective response to related pathogenic strains? (iii) Can protein fusions to Dam-repressed immunogens be used in Dam⁻ strains to elicit a protective response to heterologous antigens? (iv) Can other master switches of *in vivo* gene expression be identified, and if so, can they be used to construct novel vaccines and antimicrobials?

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REFERENCES

- Abu Kwaik, Y. & Pederson, L. L. 1996 The use of differential display-PCR to isolate and characterize a *Legionella pneumophila* locus induced during the intracellular infection of macrophages. *Mol. Microbiol.* **21**, 543–556.
- Akins, D. R., Bowrell, K. W., Caimano, M. J., Norgard, M. V. & Radolf, J. D. 1998 A new animal model for studying Lyme disease spirochetes in a mammalian host adapted state. *J. Clin. Invest.* **101**, 2240–2250.
- Braaten, B. A., Platko, J. V., Van der Woude, M. W., Simons, B. H., de Graaf, F. K., Calvo, J. M. & Low, D. A. 1992 Leucine-responsive regulatory protein controls the expression of both the *pap* and *fli* operons in *Escherichia coli*. *Proc. Natl Acad. Sci. USA* **89**, 4250–4254.
- Braaten, B. A., Nou, X., Kaltenbach, L. S. & Low, D. A. 1994 Methylation patterns in *pap* regulatory DNA control pyelonephritis-associated pili phase variation in *E. coli*. *Cell* **76**, 577–588.
- Camilli, A. & Mekalanos, J. J. 1995 Use of recombinase gene fusions to identify *Vibrio cholerae* genes induced during infection. *Mol. Microbiol.* **18**, 671–683.
- Chiang, S. L. & Mekalanos, J. J. 1998 Use of signature-tagged transposon mutagenesis to identify *Vibrio cholerae* genes critical for colonization. *Mol. Microbiol.* **27**, 797–805.
- Cormack, B. P., Gori, N. & Falkow, S. 1999 An adhesin of the yeast pathogen *Candida glabrata* mediating adherence to human epithelial cells. *Science* **285**, 578–582.
- Coulter, S. N., Schwan, W. R., Ng, E. Y. W., Langhorne, M. H., Ritchie, H. D., Westrock-Wadman, S., Hufnagle, W. O., Folger, K. R., Bayer, A. S. & Stover, C. K. 1998 *Staphylococcus aureus* genetic loci impacting growth and survival in multiple infection environments. *Mol. Microbiol.* **30**, 393–404.
- Darwin, A. J. & Miller, V. L. 1999 Identification of *Yersinia enterocolitica* genes affecting survival in an animal host using signature-tagged transposon mutagenesis. *Mol. Microbiol.* **32**, 51–62.
- Edelstein, P. H., Edelstein, M. A. C., Higa, F. & Falkow, S. 1999 Discovery of virulence genes of *Legionella pneumophila* by using signature-tagged mutagenesis in a guinea pig pneumonia model. *Proc. Natl Acad. Sci. USA* **96**, 8190–8195.
- Falkow, S. 1997 What is a pathogen? *Am. Soc. Microbiol. News* **63**, 356–359.
- Fuller, T. E., Shea, R. J., Thacker, B. J. & Mulks, M. H. 1999 Identification of *in vivo* induced genes in *Actinobacillus pleuropneumonia*. *Microb. Pathogen.* **27**, 311–327.
- Garcia-del Portillo, F., Foster, J. W., Maguire, M. E. & Finlay, B. B. 1992 Characterization of the micro-environment of *Salmonella typhimurium*-containing vacuoles within MDCK epithelial cells. *Mol. Microbiol.* **6**, 3289–3297.
- Garcia-del Portillo, F., Pucciarelli, M. G. & Casadesus, J. 1999 DNA adenine methylase mutants of *Salmonella typhimurium* show defects in protein secretion, cell invasion, and M cell cytotoxicity. *Proc. Natl Acad. Sci. USA* **96**, 11578–11583.
- Garcia Vescovi, E., Soncini, F. C. & Groisman, E. A. 1996 Mg²⁺ as an extracellular signal: environmental regulation of *Salmonella* virulence. *Cell* **84**, 165–174.
- Groisman, E. A. & Heffron, F. 1995 Regulation of *Salmonella* virulence by two-component regulatory systems. In *Two-component signal transduction* (ed. J. A. Hoch & T. J. Silhavy), pp. 319–332. Washington, DC: American Society for Microbiology Press.
- Hale, W. B., Van der Woude, M. W. & Low, D. A. 1994 Analysis of nonmethylated GATC sites in the *Escherichia coli* chromosome and identification of sites that are differentially methylated in response to environmental stimuli. *J. Bacteriol.* **176**, 3438–3441.
- Handfield, M., Sanschagrin, F. C., Cardinal, G., Mahan, M. J., Woods, D. E. & Levesque, R. C. 2000 *In vivo* induced genes and iron uptake in *Pseudomonas aeruginosa*. *Infect. Immun.* (In the press.)
- Hassan, J. O. & Curtiss III, R. 1997 Efficacy of a live avirulent *Salmonella typhimurium* vaccine in preventing colonization and invasion of laying hens by *Salmonella typhimurium* and *Salmonella enteritidis*. *Avian Dis.* **41**, 783–791.
- Heithoff, D. M., Conner, C. P., Hanna, P. C., Julio, S. M., Hentschel, U. & Mahan, M. J. 1997a Bacterial infection as assessed by *in vivo* gene expression. *Proc. Natl Acad. Sci. USA* **94**, 934–939.
- Heithoff, D. M., Conner, C. P. & Mahan, M. J. 1997b Dissecting the biology of a pathogen. *Trends Microbiol.* **5**, 463–520.
- Heithoff, D. M., Conner, C. P., Hentschel, U., Govantes, F., Hanna, P. C. & Mahan, M. J. 1999a Coordinate intracellular expression of *Salmonella* genes induced during infection. *J. Bacteriol.* **181**, 799–807.

- Heithoff, D. M., Sinsheimer, R. L., Low, D. A. & Mahan, M. J. 1999b An essential role for DNA adenine methylation in bacterial virulence. *Science* **284**, 967–970.
- Henderson, I. R., Meehan, M. & Owen, P. 1997 A novel regulatory mechanism for a novel phase-variable outer membrane protein of *Escherichia coli*. *Adv. Exp. Med. Biol.* **412**, 349–355.
- Hensel, M., Shea, J. E., Gleeson, C., Jones, M. D., Dalton, E. & Holden, D. W. 1995 Simultaneous identification of bacterial virulence genes by negative selection. *Science* **269**, 400–403.
- Hormaeche, C. E., Mastroeni, P., Harrison, J. A., Demarco de Hormaeche, R., Svenson, S. & Stocker, B. A. D. 1996 Protection against oral challenge three months after i.v. immunization of BALB/c mice with live Aro *Salmonella typhimurium* and *Salmonella enteritidis* vaccines is serotype (species)-dependent and only partially determined by the main LPS O antigen. *Vaccine* **14**, 251–259.
- Krabbe, M., Weyand, N. & Low, D. A. 2000 Environmental control of pili gene expression. In *Bacterial stress responses* (ed. G. Storz & R. Hengge-Aronis). Washington, DC: American Society for Microbiology Press. (In the press.)
- Lowe, A. M., Beattie, D. T., Deresiewicz, R. L. 1998 Identification of novel *Staphylococcal* virulence genes by *in vivo* expression technology. *Mol. Microbiol.* **27**, 967–976.
- Mahan, M. J., Slauch, J. M. & Mekalanos, J. J. 1993 Selection of bacterial virulence genes that are specifically induced in host tissues. *Science* **259**, 666–668.
- Mahan, M. J., Tobias, J. W., Slauch, J. M., Hanna, P. C., Collier, J. R. & Mekalanos, J. J. 1995 Antibiotic-based selection for bacterial genes that are specifically induced during infection of a host. *Proc. Natl Acad. Sci. USA* **92**, 669–673.
- Mahan, M. J., Slauch, J. M. & Mekalanos, J. J. 1996 Environmental regulation of virulence gene expression in *Escherichia*, *Salmonella* and *Shigella* spp. In *Escherichia coli and Salmonella. Cellular and molecular biology*, 2nd edn (ed. F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaecter and H. E. Umberger), pp. 2803–2815. Washington, DC: American Society for Microbiology Press.
- Marinus, M. G. 1996 Methylation of DNA. In *Escherichia coli and Salmonella. Cellular and molecular biology*, 2nd edn (ed. F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaecter and H. E. Umberger), p. 782–791. Washington DC: American Society for Microbiology Press.
- Mei, J. M., Nourbakhsh, F., Ford, C. W. & Holden, D. W. 1997 Identification of *Staphylococcus aureus* virulence genes in a murine model of bacteraemia using signature-tagged mutagenesis. *Mol. Microbiol.* **26**, 399–407.
- Mekalanos, J. J. 1992 Environmental signals controlling the expression of virulence determinants in bacteria. *J. Bacteriol.* **174**, 1–7.
- Merrell, D. S. & Camilli, A. 2000 The *cadA* gene of *Vibrio cholerae* is induced during infection and plays a role in acid tolerance. *Mol. Microbiol.* **34**, 836–849.
- Miller, J. F., Mekalanos, J. J. & Falkow, S. 1989 Coordinate regulation and sensory transduction in the control of bacterial virulence genes. *Science* **243**, 916–922.
- Miller, S. I., Mekalanos, J. J. & Pulkkinen, W. S. 1990 *Salmonella* vaccines with mutations in the *phoP* virulence regulon. *Res. Microbiol.* **141**, 817–821.
- Munoz, C. I. & Bailey, A. M. 1998 A cutinase encoding gene from *Phytophthora capsici* isolated by differential display RT-PCR. *Curr. Genet.* **33**, 225–230.
- Nou, X., Skinner, B., Braaten, B., Blyn, L., Hirsh, D. & Low, D. A. 1993 Regulation of pyelonephritis-associated pili phase variation in *Escherichia coli*: binding of the PapI and Lrp regulatory proteins is controlled by DNA methylation. *Mol. Microbiol.* **7**, 545–553.
- Plum, G. & Clarke-Curtiss, J. E. 1994 Induction of *Mycobacterium avium* gene expression following phagocytosis by human macrophages. *Infect. Immun.* **62**, 476–483.
- Plum, G., Brenden, M., Clarke-Curtiss, J. E. & Pulverer, G. 1997 Cloning, sequencing, and expression of the *mig* gene of *Mycobacterium avium*, which codes for a secreted macrophage induced protein. *Infect. Immun.* **65**, 4548–4557.
- Polissi, A., Pontiggia, A., Feger, G., Altieri, M., Mottle, H., Ferrari, L. & Simon, D. 1998 Large scale identification of virulence genes from *Streptococcus pneumoniae*. *Infect. Immun.* **66**, 5620–5629.
- Rainey, P. B. 1999 Adaptation of *Pseudomonas fluorescens* to the plant rhizosphere. *Environ. Microbiol.* **1**, 243–257.
- Rainey, P., Heithoff, D. M. & Mahan, M. J. 1997 Single-step conjugative cloning of bacterial fusions involved in microbial–host interactions. *Mol. Gen. Genet.* **256**, 84–87.
- Rindi, L., Lari, N. & Garzelli, C. 1999 Search for genes potentially involved in *Mycobacterium tuberculosis* virulence by mRNA differential display. *Biochem. Biophys. Res. Commun.* **258**, 94–101.
- Rivera-Marrero, C. A., Burroughs, M. A., Masse, R. A., Vannberg, F. O., Leimbach, D. L., Roman, J. & Murtagh, J. J. 1998 Identification of genes differentially expressed in *Mycobacterium tuberculosis* by differential display PCR. *Microb. Pathogen.* **25**, 307–316.
- Shea, J. E., Hensel, M., Gleeson, C. & Holden, D. W. 1996 Identification of a virulence locus encoding a second type III secretion system in *Salmonella typhimurium*. *Proc. Natl Acad. Sci. USA* **93**, 2593–2597.
- Soncini, F. C. & Groisman, E. A. 1996 Two-component regulatory systems can interact to process multiple environmental signals. *J. Bacteriol.* **178**, 6796–6801.
- Soncini, F. C., Garcia Vescovi, E., Solomon, F. & Groisman, E. A. 1996 Molecular basis of the magnesium deprivation response in *Salmonella typhimurium*: identification of PhoP-regulated genes. *J. Bacteriol.* **178**, 5092–5099.
- Staib, P., Kretschmar, M., Nichterlein, T., Kohler, G., Michel, S., Hof, H., Hacker, J. & Morschhauser, J. 1999 Host-induced, stage specific virulence gene activation in *Candida albicans* during infection. *Mol. Microbiol.* **32**, 533–546.
- Tavazoie, S. & Church, G. M. 1998 Quantitative whole-genome analysis of DNA–protein interactions by *in vivo* methylase protection in *E. coli*. *Nat. Biotechnol.* **16**, 566–571.
- Valdivia, R. H. & Falkow, S. 1996 Bacterial genetics by flow cytometry: rapid isolation of *Salmonella typhimurium* acid-inducible promoters by differential fluorescence induction. *Mol. Microbiol.* **22**, 367–389.
- Valdivia, R. H. & Falkow, S. 1997 Fluorescence-based isolation of bacterial genes expressed within host cells. *Science* **277**, 2007–2011.
- Van der Woude, M. W. & Low, D. A. 1994 Leucine-responsive regulatory protein and deoxyadenosine methylase control the phase and expression of the *sfa* and *daa* pili operons in *Escherichia coli*. *Mol. Microbiol.* **11**, 605–618.
- Van der Woude, M. W., Braaten, B. A. & Low, D. A. 1992 Evidence for global regulatory control of pilus expression in *Escherichia coli* by Lrp and DNA methylation: model building based on analysis of *pap*. *Mol. Microbiol.* **6**, 2429–2435.
- Van der Woude, M., Braaten, B. & Low, D. A. 1996 Epigenetic phase variation of the *pap* operon in *Escherichia coli*. *Trends Microbiol.* **4**, 5–9.
- Van der Woude, M., Hale, W. B. & Low, D. A. 1998 Formation of DNA methylation patterns: nonmethylated GATC sequences in the *gut* and *pap* operons. *J. Bacteriol.* **180**, 5913–5920.

- Waldor, M. K. & Mekalanos, J. J. 1996 Lysogenic conversion by a filamentous phage encoding cholera toxin. *Science* **272**, 1910–1914.
- Wang, J., Mushegian, A., Lori, S. & Jin, S. 1996 Large scale isolation of candidate virulence genes of *Pseudomonas aeruginosa* by *in vivo* selection. *Proc. Natl Acad. Sci. USA* **93**, 10 434–10 439.
- Young, G. & Miller, V. L. 1997 Identification of novel chromosomal loci affecting *Yersinia enterocolitica* pathogenesis. *Mol. Microbiol.* **25**, 319–328.
- Yuk, M. H., Harvill, E. T. & Miller, J. F. 1998 The *bugAS* virulence control system regulates type III secretion in *Bordetella bronchiseptica*. *Mol. Microbiol.* **28**, 945–959.
- Zhao, H., Li, X., Johnson, D. E. & Mobley, H. L. 1999 Identification of protease and *rpoN*-associated genes of uropathogenic *Proteus mirabilis* by negative selection in a mouse model of ascending urinary tract infection. *Microbiology* **145**, 185–195.