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# *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.* 1997*b*). 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. $LD_{50}$). 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 microenvironments 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.* 1999*a*; 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.* 1997*b*). 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*

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

(*Cont*.)

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Table 1 (*Cont.*)

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

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Table 1 (*Cont.*)

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



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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.* 1999*a*). 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.

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## 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.* 1999*b*). 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.* 1999*b*). 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 *Mbo*I 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.* 1999*b*). 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.* 1999*b*). 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 $LD_{50}$ of virulent *Salmonella* (Heithoff *et al.* 1999*b*), 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.* 1999*b*). 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.* 1999*b*). If

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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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