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THE ROYAL SOCIETY

Detection and analysis of gene expression during infection by *in vivo* expression technology

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Many limitations associated with the use of *in vitro* models for study of bacterial pathogenesis can be overcome by the use of technologies that detect pathogen gene expression during the course of infection within an intact animal. *In vivo* expression technology (IVET) accomplishes this with versatility: it has been developed with a variety of reporter systems which allow for either *in vivo* selection or *ex vivo* screening. Selectable gene fusion systems generally allow for the complementation of a bacterial metabolic defect that is lethal *in vivo*, or for antibiotic resistance during the course of *in vivo* antibiotic challenge. In contrast, the screenable gene fusion system uses a site-specific DNA recombinase that, when expressed *in vivo*, excises a selectable gene cassette from the bacterial chromosome. Loss of this cassette can then be either screened or selected for *ex vivo*. The recombinase-based IVET can be used to detect genes that are transcriptionally induced during infection, including those expressed transiently or at low levels and, in addition, can be used to monitor the spatial and temporal expression of specific genes during the course of infection.

Keywords: IVET; RIVET; *Vibrio cholerae*; *cadA*; *ctxA*; *tcpA*

1. INTRODUCTION

During the 20th century a wealth of knowledge has been garnered concerning pathogenic microbes and the mechanisms whereby they cause disease. Empirical lines of research and epidemiological studies have yielded valuable information, which has been applied to the development of vaccines and antimicrobial compounds and the control of some microbes within their natural reservoirs. Unfortunately, an increase in the number of antibiotic-resistant strains of most pathogenic bacterial species has become a severe problem for the treatment of disease within the general population and also the prevention of disease in immunocompromised people. Due to this, the scientific community has found itself faced with the need for development of novel antimicrobials and vaccines, and for accompanying discoveries in the basic sciences which can aid in these pursuits.

Two areas of basic research of fundamental importance are achieving a complete understanding of the physiology of pathogens during infection and the pathogenic mechanisms they employ at each stage of infection. One can imagine that distinct but partially overlapping sets of bacterial factors are required and produced at each stage of infection. For instance, the stages of infection for intestinal pathogens involve (i) entry, (ii) circumvention or survival in the face of physical barriers such as gastric acidity, (iii) primary attachment to host cells or tissue, (iv) invasion into host cells or tissues (in the case of invasive pathogens) or increased adherence by extracellular pathogens, (v) avoidance of and/or resistance to host innate immune defences, (vi) acquisition of nutrients and multiplication, and (vii) evacuation from the host to either a new host or an environmental reservoir. When considered as a whole, the prospect of understanding all of the factors produced and required by pathogens to accomplish these diverse processes becomes not only ambitious, but daunting.

Past attempts to identify and understand the function of pathogenicity factors have centred around the ability to stimulate expression of these factors *in vitro* using tissue culture cells or abiotic conditions which usually, but not always, mimic host signals. For instance, knowledge that classical biotype *Vibrio cholerae* produces cholera toxin (CT) under growth conditions of low pH and non-physiological low temperature (30 °C), led to a number of elegant studies that have identified other pathogenicity factors that are co-regulated with CT (Peterson & Mekalanos 1988; Taylor *et al*. 1986) or sensory and regulatory factors that serve to regulate these pathogenicity factors (DiRita *et al*. 1991; Miller *et al*. 1987). Similar *in vitro* strategies have been employed to study many other pathogenic organisms, such as *Bordetella pertussis* (Finn *et al*. 1991) and *Salmonella typhimurium* (Valdivia & Falkow 1996). While such *in vitro* studies have been fruitful in the past, their potential use in developing a complete and accurate understanding of the factors elicited during the course of a bona fide infection is severely limited by our inability to reproduce the complex and dynamic environmental stimuli that are present *in vivo*. For instance, it is unlikely that studies done *in vitro*, including tissue culture models of infection,

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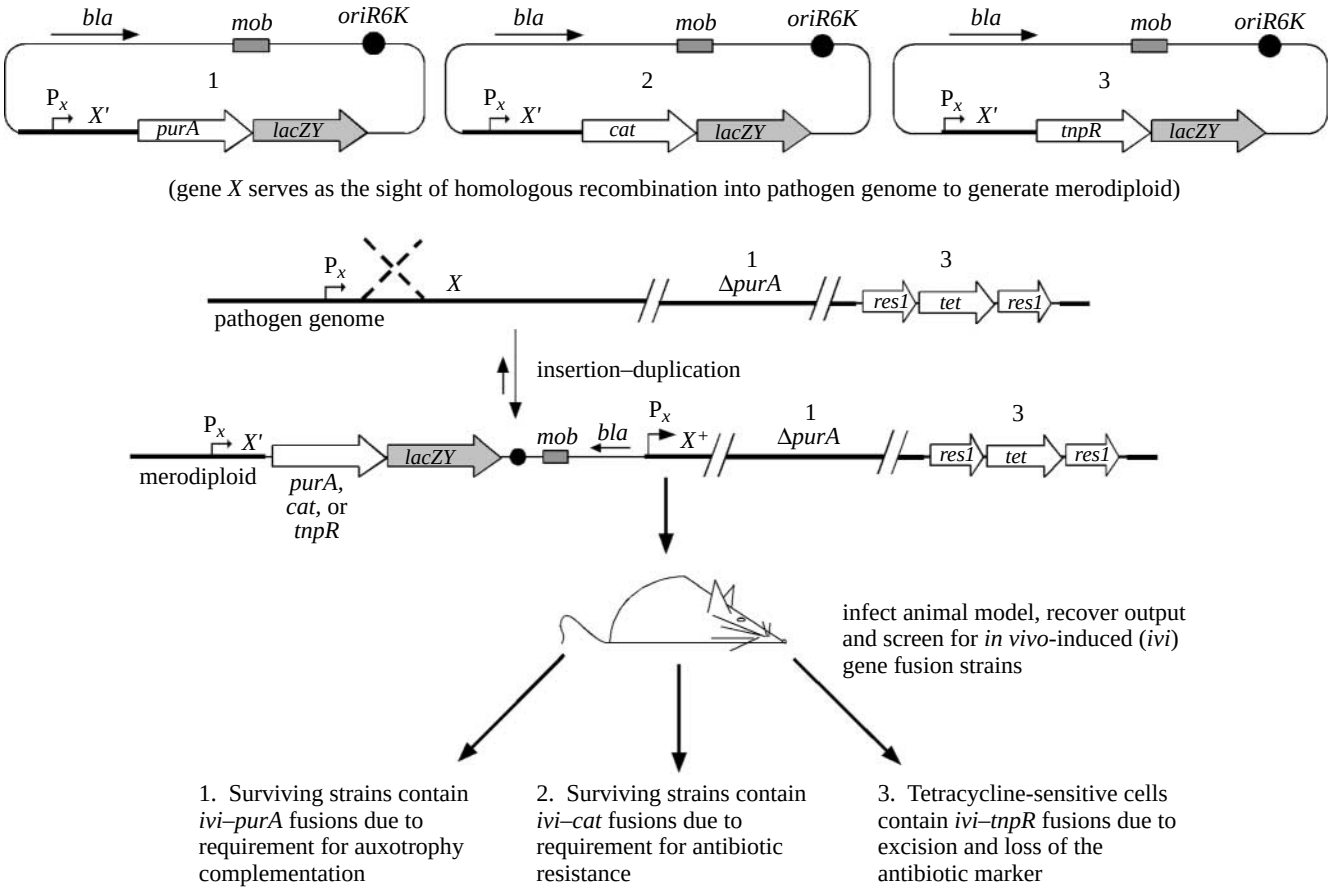

Figure 1. Graphic depiction of three variations of IVET. As shown, auxotrophy complementation IVET selections are conducted using fusions to a promoterless *purA* gene (plasmid 1), antibiotic IVET selections are conducted using fusions to a promoterless antibiotic gene such as *cat* (plasmid 2), and recombinase-based *in vivo* expression technology (RIVET) screening is done using a promoterless *tnpR* allele (plasmid 3), which when produced, will cleave a Tc^r gene from elsewhere in the bacterial genome. Reporter gene fusion libraries are constructed by ligating random genomic fragments (designated as gene *X'*) into the IVET vector of choice, followed by transformation into the pathogen of interest. The suicide plasmids then recombine into the chromosome by insertion–duplication creating a merodiploid. In the case of RIVET, a prescreen is required to remove strains harbouring *in vitro* active gene fusions: this is accomplished by selecting for Tc^r, $LacZ^-$ colonies. In all cases, fusion strains are passaged through an appropriate animal model of disease and collected from infected tissues and/or fluids after a period of time. In the case of the antibiotic-based IVET, the antibiotic (in this example, Cm) must be present at sufficient concentrations in animal tissues to select for *in vivo* expression of the gene fusion. Strains containing infection-induced gene fusions to *purA* and *cat* are selected in the host and are subsequently screened for lack of *in vitro* expression on LacZ indicator plates. In contrast, infection-induced gene fusions to *tnpR* are screened for, post-infection, by virtue of Tc^s and lack of expression on LacZ indicator plates.

will reveal the identities and modes of action of pathogenicity factors that play important roles in circumventing or resisting host humoral immunity. However, development and recent advances made in *in vivo* expression technology (IVET) have provided powerful genetic tools for identifying and studying bacterial genes that are induced during infection.

IVET is designed as a promoter trap method whereby random genomic fragments are ligated in front of a promoterless reporter gene (figure 1). Reporter activity can then be used as an indication of transcriptional activity of the fused gene. IVET was first used to identify *in vivo*-induced (*ivi*) genes in a mammalian pathogen in 1993 (Mahan *et al.* 1993). Note that a similar strategy, based on antibiotic selection, was first used by Osbourn *et al.* (1987) to identify virulence genes in the plant pathogen *Xanthomonas campestris*. The initial IVET strategy as developed by Mahan and colleagues, relied upon the fact that purine auxotrophs of *S. typhimurium* are unable to survive passage through a mouse after intraperitoneal inoculation. Transcriptional fusions to a promoterless *purA–lacZY* synthetic operon were constructed and integrated into an *S. typhimurium* strain in which the chromosomal *purA* gene had been deleted (figure 1). Only fusions which were active during infection would result in expression of *purA*, and allow survival and multiplication of the strain in the animal. Most such strains had fusions that were also active during *in vitro* growth, i.e. these strains had constitutively active gene fusions. However, the subset of strains containing *ivi* gene fusions could easily be identified by blue–white screening of output colonies on media containing 5-bromo-4-chloro-3-indolyl-β-D-galactoside due to the bicistronic nature of the *purA–lacZY* fusion. Output strains that were $LacZ^-$ were said to contain gene promoters driving expression of *purA–lacZY* that were specifically active within the host environment.

The auxotroph complementation IVET strategy has now been used in a number of pathogenic species.

Perhaps the most successful application involved large-scale screening of promoter fusions in *S. typhimurium* for induction in BALB/c mice and in cultured murine macrophages (Mahan *et al.* 1993; Heithoff *et al.* 1997). To date, over 100 genes have been found that are induced within these environments. While the identities of many of these genes have not been reported, those that have been reported fall into four broad categories: (i) regulators, (ii) metabolic/physiological, (iii) stress response, and (iv) unknown function. Similar screens have been conducted to identify *Pseudomonas aeruginosa* genes that are induced during infection of neutropenic mice and in response to exposure to respiratory mucus from cystic fibrosis patients, and in the plant pathogen *P. fluorescens* in response to rhizosphere colonization (Wang *et al.* 1996*a*,*b*; Rainey 1999). Each of these studies has revealed similar classes of genes and together they have proven that IVET is a powerful tool for the identification of *ivi* genes.

In the past six years, the original IVET strategy has been modified to include two additional selection strategies (figure 1). Each of these modifications has increased the potential application of IVET to include more diverse organisms and, theoretically, to identify a more diverse set of *ivi* genes within each organism. The first reporter modification applied to IVET involved the replacement of *purA* with *cat* (Mahan *et al.* 1995). Active promoters fused to the *cat* gene convey resistance to chloramphenicol (Cm). Therefore, strains containing random promoter fusions can be passaged through antibiotic-treated animals or tissue culture models to select promoter fusions that are active *in vivo*. This modification should expand the potential applications of IVET to include pathogens for which knowledge of virulence-attenuating auxotrophies is not known or is difficult to derive.

Antibiotic-based IVET strategies have been used in *S. typhimurium*, *Yersinia enterocolitica* and most recently in *Streptococcus gordonii* (Mahan *et al.* 1995; Young & Miller 1997; Kilic 1999). IVET screens conducted in *Y. enterocolitica* by Young & Miller provided the first large-scale screen for genes involved in *Yersinia* pathogenesis. In this study, *Y. enterocolitica* strains containing random transcriptional fusions to a promoterless *cat* gene were first selected for their ability to survive passage through a Cm-treated mouse and then screened for their inability to grow on both rich and minimal media containing Cm. In this manner, they were able to eliminate constitutively expressed genes, genes involved in nutrient uptake, and genes involved in metabolic functions. The remainder of fusions could then be said to identify virulence-associated genes that were specifically induced upon exposure to host stimuli. Once again, a broad class of genes was identified and could be subdivided into general categories: (i) stress response, (ii) response to iron starvation, (iii) cell envelope maintenance, and (iv) unknown functions.

The second reporter modification applied to IVET involved construction of a promoterless *tnpR–lacZY* reporter. The *tnpR* gene codes for the site-specific DNA resolvase from Tn$\gamma\delta$. The resolvase enzyme is able to mediate recombination between two directly repeated copies of a specific target DNA sequence, called *res1* sites. These *res1* sites are inserted, flanking a reporter gene, within the chromosome of the pathogen of interest (figure 1). If a particular *tnpR* fusion is transcriptionally induced during infection, even transiently and/or at a low level, the resolvase that is produced will catalyse a permanent and heritable change in the bacterium by excising the reporter from the chromosome. Resolved strains can be screened or selected after recovering the bacteria from host tissues. For instance, in the original study describing the recombinase-based IVET (RIVET), induced resolvase fusions catalysed the excision of a tetracycline-resistance (Tc^r) gene from the chromosome, resulting in conversion of the fusion strain to a Tc^s phenotype (Camilli *et al.* 1994). The strains harbouring induced fusions were then identified by replica-plating output colonies onto an agar medium supplemented with Tc.

RIVET has been applied to discover *ivi* genes in *V. cholerae* and *Staphylococcus aureus*. In the first implementation of RIVET for this purpose, over a dozen *V. cholerae* genes were identified for which the levels of transcription increased during infection of the small intestine of suckling mice (Camilli & Mekalanos 1995). Most recently, RIVET was used to identify *ivi* genes in *S. aureus* using a murine renal abscess model of disease. This study represents the first application of any IVET strategy to a Gram-positive organism, and showed its versatility as 45 staphylococcal genes were identified that were induced specifically upon infection (Lowe *et al.* 1998). Once again, a broad class of genes was identified, of which only six genes were previously known. Eleven genes were shown to have homology to non-staphylococcal genes and the others showed no homology to sequences within available databases. In addition, it was shown that mutations within seven of the identified *ivi* genes resulted in significant attenuation of pathogenicity compared with the wild-type, exemplifying the fact that IVET can identify *ivi* genes that encode essential pathogenicity factors.

Overall, IVET has proven itself as a valuable method for the identification of genes that are induced *in vivo*. In addition, it has proven applicable to a variety of different pathogenic species of bacteria. Despite their strengths, though, each of the IVET strategies has characteristics that limit the types of gene that will be identified using that strategy. For instance, for the auxotrophy complementation method, it is essential that the chosen selectable gene be required for growth in the same host compartment that the pathogenicity gene (to be identified) is expressed in. Thus, a limitation of this IVET strategy is that if the selectable gene is required throughout most of the infectious process, then preferential enrichment will occur for strains harbouring gene fusions that are constitutively and highly expressed in the host animal. Thus, pathogenicity genes that are expressed at only one stage of infection, or that are transcribed at low levels, may not be identifiable via this approach.

The antibiotic selection, while increasing the applicability of IVET, is more complicated since a suitable antibiotic must be administered and proper concentrations maintained in infected host tissues. Moreover, administration of antibiotics may disrupt the natural course of an infection and thus may not allow a completely unbiased look at gene induction. On the other hand, the former characteristic can provide some flexibility in designing a selection. For example, administering the antibiotic at only one stage of infection, or in

one host compartment, should facilitate identification of pathogenicity factors that may be expressed at only one stage of infection, or in only one host compartment, respectively. In addition, it is likely that the concentration of the antibiotic can be lowered to allow identification of *ivi* genes that are transcribed at low levels *in vivo*.

RIVET-based screens suffer from two primary limitations. First, because the site-specific DNA recombinase acts efficiently at only one substrate sequence, a low level of expression of the recombinase gene fusion is sufficient to catalyse the excision event. Although this exquisite sensitivity allows detection of transient and/or low-level gene induction events, it unfortunately prohibits the identification of pathogenicity genes that have high basal levels of transcription during *in vitro* growth. This is due to the fact that a high basal level of transcription results in a strain that is unable to be constructed in the unresolved state. Second, *ex vivo* screening of strains containing active fusions has proven laborious as conversion of the strain from Tc^r to Tc^s is screened for by replica-plating. However, recent modifications have largely overcome both of these limitations, and these modifications will be discussed below along with an in-depth analysis of applications of RIVET to study the diarrhoeal pathogen *V. cholerae*.

2. APPLICATION OF RIVET TO *V. CHOLERAE*

RIVET was first developed to study the intestinal pathogen *V. cholerae* (Camilli *et al*. 1994). *V. cholerae* is a Gram-negative facultative pathogen that is the causative agent of the epidemic and endemic diarrhoeal disease cholera. After ingestion in contaminated water or food, *V. cholerae* transits the gastric acid barrier and colonizes the relatively sterile brush border of the small intestine. At this point, *V. cholerae* resists innate immune defences, acquires nutrients, multiplies prodigiously and produces CT, the toxic activity of which is the major cause of the profuse watery diarrhoea that ensues (for a review, see Wachsmuth *et al*. 1994). CT is an A-B-subunit toxin, which causes increased secretion of electrolytes and water into the lumen of the intestine (Sears & Kaper 1996). Additional pathogenicity factors have been identified by their coordinated *in vitro* regulation with CT. Toxin-coregulated pilus (TCP) is probably the best studied of these. This type IV bundle-forming pilus is absolutely essential for intestinal colonization in human and animal models of cholera (Herrington *et al*. 1988; Taylor *et al*. 1986), although its precise role remains unknown.

To begin to understand the intestinal physiology of *V. cholerae* and to increase our knowledge of its pathogenicity, RIVET technology has been used to identify genes that are induced within the suckling mouse and within the rabbit ligated ileal loop models of cholera (Camilli & Mekalanos 1995; Merrell & Camilli 1999). In the first of these studies, approximately 13 000 strains containing fusions that were inactive during laboratory growth on a rich agar medium (Luria–Bertani (LB)) (as assessed by their $LacZ^-$ and Tc^r phenotypes, see figure 1) were intragastrically inoculated into suckling CD-1 mice. After 24 h, mice were killed, and bacteria were recovered from homogenates of the small intestines by plating on LB agar. Those strains containing fusions that were induced during infection were screened for by replica-plating colonies to LB supplemented with Tc (Camilli & Mekalanos 1995). A similar strategy was used for screens conducted within rabbit ligated loops (Merrell & Camilli 1999). In both studies, recovered strains that were now Tc^s could be said to contain fusions that were specifically activated *in vivo*. Both of these screens were successful and identified genes that fall into several categories: (i) metabolic, (ii) adaptive, (iii) nutrient scavenging, (iv) motility, and (v) unknown functions (table 1).

Table 1. V. cholerae *infection-induced genes identified through RIVET*

gene	function
metabolic genes	
cysI	cysteine biosynthesis
argA	arginine biosynthesis
sucA	TCA cycle enzyme
nirT	nitrite reductase
adaptation genes	
cadA	acid tolerance
vieB	response regulator
nutrient scavenging genes	
hlyC	secreted lipase
xds	secreted DNase
iviVI	ABC transporter
motility genes	
α-flaA	flagellin antisense
α-cheV	chemotaxis protein antisense
iviV	chemoreceptor
genes of unknown function	
iviX	—
iviXI	—
iviXIII	—

3. GENES INVOLVED IN METABOLISM

Identification of genes that are involved in metabolic pathways has been a common theme of every IVET search conducted thus far. This class of genes is often, unfortunately, considered uninteresting due to the fact that they are involved in so-called 'housekeeping' functions, and do not encode toxins or other factors which interact directly with the host. Perhaps this bias is due partly to the preconception that study of metabolic factors of pathogens will probably reveal nothing about the biology of the host. The fact that so many IVET searches reveal metabolic genes that are induced within the host environment, perhaps, should sound a cautionary note to the scientific community about what we consider interesting. There is, indeed, much to be learned about the growth physiology of pathogens within host environments, and these findings will in turn directly reflect upon the nature of the host compartments or tissues in which the pathogens reside. Moreover, in keeping with one of the primary goals of medical microbiological research, i.e. to develop safe and effective antimicrobials, it is worth noting that the majority of currently used antibiotics target biosynthetic and metabolic pathways (e.g. β-lactams, aminoglycosides, sulphonamides).

Among the *V. cholerae ivi*-encoded factors identified using RIVET are CysI, which catalyses an intermediate

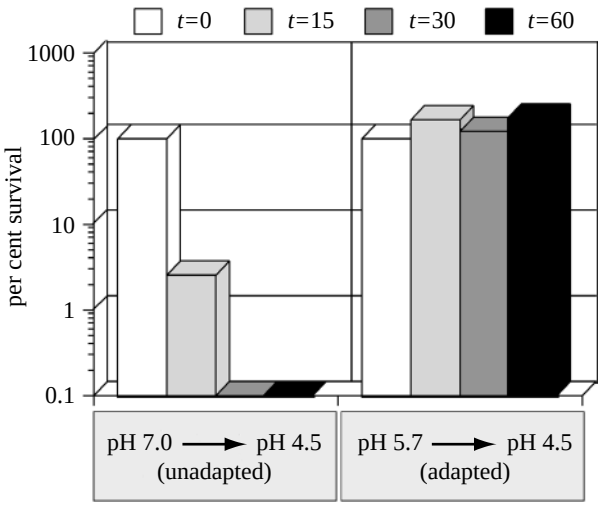

Figure 2. The *V. cholerae* ATR to inorganic acid challenge. Wild-type *V. cholerae* was either unadapted by growth in LB pH 7.0, or adapted for 1h by growth in LB pH 5.7 prior to acid shock in LB pH 4.5. The per cent survival at 15, 30 and 60 min was calculated by comparison with the initial numbers of colony-forming units (CFU) measured at time 0. Adapted from Merrell & Camilli (1999).

step in the biosynthesis of L-cysteine from inorganic sulphate; SucA, which is part of a multisubunit enzyme that catalyses a step in the tricarboxylic acid cycle whereby α-ketoglutarate is oxidatively decarboxylated to succinyl CoA and carbon dioxide; NirT, which codes for a tetrahaem nitrite reductase and that probably takes part in respiratory nitrate reduction; and ArgA, which catalyses the first step in the biosynthesis of L-arginine. *CysI*, *sucA*, *nirT* and *argA* were all found to be transcriptionally induced, to varying degrees, during the course of infection in the suckling mouse model, which suggested that these genes play roles in growth or survival during the course of infection. However, reduced pathogenicity, as assessed by competition assays in suckling mice, only accompanied a null mutation in *argA*, and not in the other three *ivi* genes (Camilli & Mekalanos 1995). Thus, members of this latter group of genes either do not fulfil an essential role during infection or there is redundancy of function. The identities of these *ivi* genes and knowledge of their enzymatic functions, nevertheless, provide useful information concerning the nutritional and physiological status of the mouse small intestinal environment. For example, the site(s) colonized by *V. cholerae* appears to be limiting for both L-cysteine and L-arginine. Interestingly, induction of the *Listeria monocytogenes arpJ* gene encoding a subunit of an L-arginine transporter was transcriptionally induced during infection of a tissue culture cell line (Klarsfeld *et al*. 1994). Thus, perhaps exogenous L-arginine, which is an essential amino acid supplied only through diet in humans and rodents, is of limited availability to bacterial pathogens in a variety of host tissues.

The frequent identification of *ivi* genes that appear to be non-essential for virulence points to the possible existence of overlapping or redundant pathways: these possibilities are often poorly anticipated or are overlooked. Bacteria have shown themselves to be particularly flexible and often possess a variety of back-up strategies to survive stresses and obtain essential nutrients. It is perhaps these redundant pathways that should be more frequently studied and subsequently targeted for vaccine and antimicrobial drug development.

4. GENES INVOLVED IN ADAPTATION

The necessity to adapt to rapidly changing environments is of great importance to facultative pathogens. For instance, the natural reservoir for *V. cholerae* is aquatic, brackish water environments. This organism is capable of forming close associations with numerous planktonic micro-organisms (Colwell & Huq 1994). Indeed, algal blooms have been linked to seasonal outbreaks of cholera in endemic areas of the world. Although the environmental conditions experienced by *V. cholerae* in its natural reservoir probably differ substantially from those in the human intestinal tract, *V. cholerae* has evolved the capacity to infect its human host directly from environmental reservoirs. Within minutes after ingestion, the bacteria must transit the gastric acid barrier and colonize the small intestine. Each step of this life cycle, no doubt, requires a variety of genes whose products play roles in adaptation. Two such adaptation-associated genes that are induced during infection have been identified using RIVET.

Escherichia coli CadA is an inducible lysine decarboxylase encoded by the second gene of the bicistronic *cadBA* operon (Meng & Bennett 1992). Decarboxylation of lysine produces cadaverine and carbon dioxide, and the reaction consumes a cytoplasmic proton. Cadaverine is then transported from the cell via the *cadB*-encoded lysine/cadaverine antiporter. It was hypothesized by Gale & Epps (1942) that the inducible amino-acid decarboxylases of *E. coli* play a role in surviving exposure to acidic environments. Indeed, it has subsequently been shown that CadA, Adi (an inducible arginine decarboxylase) and GadC (a putative glutamate/γ-amino butyrate antiporter) play roles in a physiological adaptation process known as the acid-tolerance response (ATR) (Hersh *et al*. 1996; Lin *et al*. 1995; Park *et al*. 1996). ATR is an adaptive response whereby cells that have been exposed to a mildly acidic pH, prior to exposure to very acidic pH, show a much higher per cent survival than those that are placed directly in the very acidic pH environment (Foster & Hall 1991). The ATR of *S. typhimurium* has been extensively studied, and shown to consist of a complex network of inducible acid-survival systems that are growth-phase dependent.

The *V. cholerae cadA* homologue was identified as an *ivi* gene within both the rabbit ileal loop and within the suckling mouse models of cholera (Merrell & Camilli 1999). *In vitro* analyses revealed that *cadA* transcription could be induced in a number of ways. Growth in oxygen-limiting conditions, acidic pH, and high L-lysine concentrations were all shown to be able to increase expression of *cadA*. Determination of these parameters, combined with the fact that CadA is involved in the ATR of *S. typhimurium* led us to test and show that *V. cholerae*, which had heretofore been described as acid sensitive, was able to mount a robust ATR (figure 2). Adaptation at a sublethal pH of 5.7 for 1 h endowed *V. cholerae* with resistance to killing at pH 4.5. As *V. cholerae* encounters not only low pH, but harmful organic acids during the course

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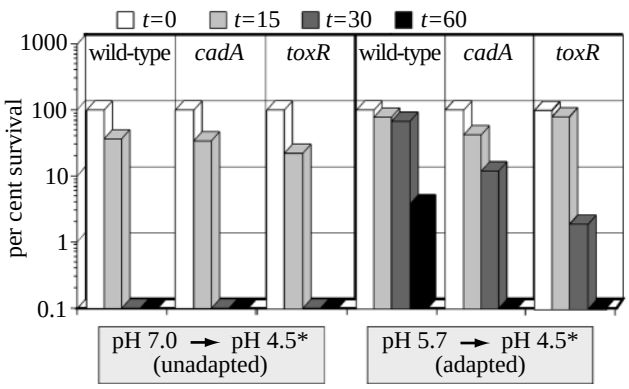

Figure 3. The ATR of *V. cholerae* wild-type, *cadA* and *toxR* mutant strains to organic acid challenge. *V. cholerae* was either unadapted by growth in LB pH 7.0, or adapted for 1h by growth in LB pH 5.7 containing 6.5 mM acetate, 1.9 mM butyrate and 2.8 mM propionate. Cells were then acid shocked in LB pH 4.5 plus 8.7 mM acetate, 2.5 mM butyrate and 3.7 mM propionate organic acids. The per cent survival at 15, 30 and 60 min was calculated by comparison with the initial numbers of CFUs measured at time 0. The asterisks indicate the addition of organic acids. Adapted from Merrell & Camilli (1999).

of its transit and colonization within the stomach and small intestine, respectively, studies were conducted that divided the ATR into two separate branches; inorganic acid (low pH) and organic acids (low pH plus the intestinal acids propionate, acetate and butyrate). CadA was shown to play a crucial role in both branches, while the major virulence regulator ToxR was shown to be necessary for organic, but not inorganic ATR (figure 3).

The relative importance of the *V. cholerae* ATR in the establishment and the progression of human infection is not known at present. It has been shown that mutant strains of *L. monocytogenes*, *S. typhimurium* and *Helicobacter pylori* that are compromised in their ability to survive exposure to acids are attenuated for pathogenicity. However, a *V. cholerae cadA* mutant was found to be unaffected in its ability to colonize the suckling mouse intestine. Conversely though, wild-type *V. cholerae* cells that were acid tolerized prior to infection were shown to have a reduced infectious dose in suckling mice (Merrell & Camilli 1999). These findings once again point to the potential for redundancy in important functions. The fact that a *cadA* mutant is not attenuated *in vivo*, yet acid-tolerized cells are more virulent, suggests that the ATR is multifactorial and partially redundant. Indeed, it has been shown that there are more than 50 proteins induced in *S. typhimurium* upon exposure to acid, and it is reasonable to predict, *a priori*, that a subset of these may fulfil redundant functions (Foster 1991). It is also interesting to speculate that acid adaptation may decrease the infectious dose in humans, aiding in the epidemic spread of cholera. Specifically, if bacteria that are shed from the human intestine are in an acid-tolerant state, and if this state persists within contaminated waters for a period of time, subsequent infections would require a lower infectious dose thus aiding transmission of *V. cholerae*.

The importance of two-component signal transduction systems whereby a 'sensor kinase' protein monitors the environment and, upon proper stimulation, transmits a signal to a 'response regulator' protein to induce an adaptative response, is probably best highlighted by the commonality and prevalence of these systems within facultative bacterial pathogens. *VieB* (previously designated *iviVII*) was identified as an *ivi* gene in *V. cholerae* within the suckling mouse model of cholera (Camilli & Mekalanos 1995). Subsequent analysis revealed that *vieB* lies within a three gene locus that encodes a sensor kinase (VieS) and two distinct response regulator proteins (VieA and VieB). VieS contains a phosphoreceiver and two transmitter domains and is thus a member of the complex sensor kinase family exemplified by BvgS (Uhl & Miller 1996) and ArcS (Georgellis *et al.* 1997). VieA is a typical response regulator, containing an N-terminal phosphoreceiver domain and a C-terminal DNA-binding domain. In contrast, VieB is atypical because, although it contains an N-terminal phosphoreceiver domain, it lacks a C-terminal DNA-binding domain. Because *vieB* is transcribed only during infection, specifically at one particular stage of infection (after colonization of the epithelium (Lee *et al.* 1998, and see below), it has been suggested that it may play an adaptive role in this particular host niche. However, such a role has been difficult to establish experimentally, as a strain containing a null mutation in *vieB*, or one containing a deletion of all three *vie* genes, retains full virulence (Lee *et al.* 1998). Hence, either the Vie system does not play a role in adaptation, the adaptive response mediated by Vie is not required for mouse infection, or there is redundant regulation of the adaptive response. Identification of the set of genes regulated by the Vie proteins should allow specific tests of each of these possibilities to be conducted. For example, and hypothetically, if null mutations in one or more Vie-induced genes were shown to attenuate virulence, then the redundant regulation hypothesis would be supported.

During the course of infection, *V. cholerae* must encounter a variety of biochemical and nutritional parameters that are characteristic of the microenvironments through which it passes or takes up residence. Since *vieB* is induced at a particular stage of infection, its transcription must be modulated by one or more of these parameters. Despite this, extensive attempts to induce *vieB* transcription *in vitro* were unsuccessful: this failure exemplifies the notion that use of *in vitro* systems designed to mimic host parameters can be limiting due to the fact that some genes may require multiple (or cryptic) *in vivo* signals to stimulate their transcription. Hence, identification of genes like *vieB*, where no tested signal has been able to stimulate *in vitro* transcription, highlights the potential of technologies such as IVET as a means of dissecting the complexity of host–pathogen interactions.

Besides being useful for identifying *ivi* genes, RIVET has an additional functionality that other methods, including IVET, lack—the ability to easily monitor the temporal and spatial patterns of *ivi* gene induction in the host. *VieB* is the first gene for which transcription during the course of infection in an intact animal was monitored (Lee *et al.* 1998). The temporal pattern of *vieB* induction was accomplished by infecting a group of animals with a *vieB::tnpR* fusion strain and then recovering bacteria from the small intestine at different times. The percentage of bacterial cells in each sample that had resolved was measured by scoring for loss of the marker gene (*tet*) within the excisable cassette. It was determined

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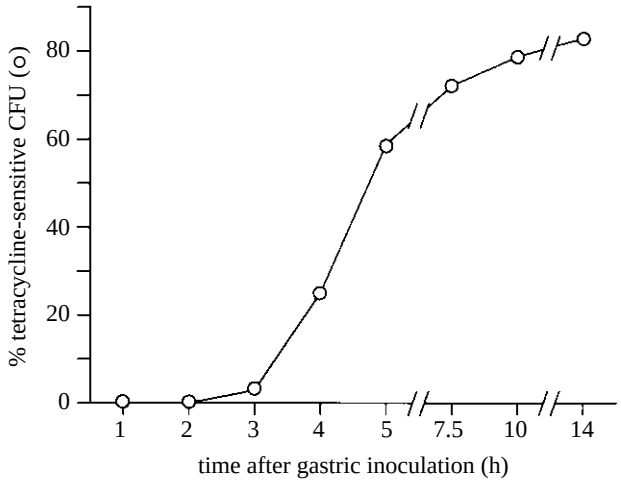

Figure 4. Kinetics of transcriptional induction of *vieB* during infection. *V. cholerae vieB::tnpR* was used to intragastrically inoculate suckling CD-1 mice. At the post-inoculation times indicated on the *x*-axis, the small intestines were removed and homogenized. The per cent Tc^s CFUs per intestine, shown on the *y*-axis, was determined by replica-plating colonies. Adapted from Lee *et al.* (1999).

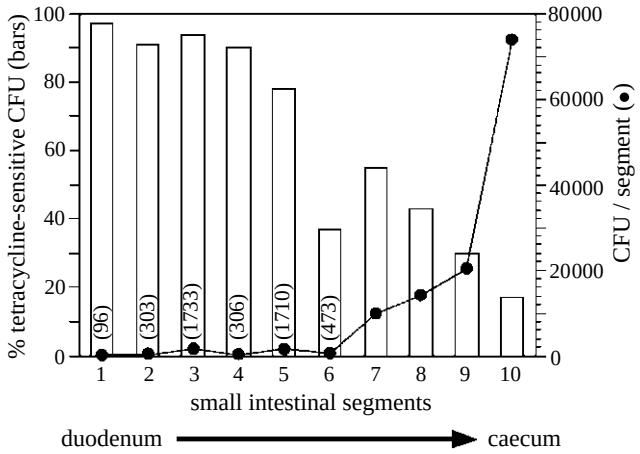

Figure 5. Spatial pattern of *vieB* transcription induction in the small intestine. *V. cholerae vieB::tnpR* was used to intragastrically inoculate a suckling CD-1 mouse. At 3.5 h post-inoculation the small intestine and caecum were removed and the former was dissected into nine segments of equal length. The caecum was considered the tenth segment. For each segment, the total CFUs (shown on the right axis) and per cent Tc^s CFU (shown on the left axis) were determined. The total CFU for segments 1–6 are shown in parentheses above each data point. Adapted from Lee *et al.* (1999).

that *vieB* transcription was induced approximately 3–4 h post-inoculation (figure 4).

Once the time of *vieB* induction was known, the spatial pattern of induction in the small intestine at this time could be determined. This procedure involved removing and dissecting the small intestine and caecum into ten segments of equal length at 3.5 h post-inoculation, recovering bacteria from each segment, and replica-plating colonies to determine the extent of resolution. Resolved (Tc^s) cells were found throughout the small intestine, but with a gradient from high to low levels of resolution corresponding to the proximal to distal segments (figure 5). In this experimental system the bolus of the bacterial inoculum travels down the lumen of the small intestine during the first 3–4 h with only a minority of cells adhering to the intestinal wall (figure 5, solid circles). Three conclusions were drawn from these data. First, because the majority of bacteria that had flowed down the lumen to the ileum by 3.5 h remained unresolved, *vieB* induction was not occurring in the lumen of the stomach or duodenum. Second, the duodenum must contain a *vieB*-inducing environment, and it remains possible that more downstream segments do as well. Third, because the highest percentage of resolved cells was observed for the minority of bacteria that had colonized the duodenal epithelium, either *vieB* induction occurs during the act of colonization or soon thereafter.

The exact host signals necessary for transcriptional induction of *vieB* have not been determined, but it has subsequently been shown that strains that are blocked for the ability to colonize (via a mutation in *tcpA*) never induce *vieB* transcription *in vivo*. This result suggests that a colonization-specific signal is sent after attachment to the host mucosa (S. Lee and A. Camilli, unpublished data). The flexibility and usefulness of RIVET for determining the sites, times and requirements for gene induction *in vivo* is exemplified by these *vieB* studies. With advancements recently made in RIVET (discussed in § 8), virtually any gene that is transcriptionally induced *in vivo*, no matter how high its basal level of *in vitro* transcription, can be analysed during the course of infection.

5. GENES INVOLVED IN NUTRIENT SCAVENGING

Once a pathogen enters its host it must not only survive the onslaught of innate immune mechanisms, but must also be able to acquire nutrients that may be in limited quantity or, in some cases, quite different in composition from the previous environment. It is therefore not surprising that pathogenic species of bacteria have evolved the ability to acquire nutrients in diverse host environments. One would expect, for this reason, that one prominent subclass of RIVET-identified genes will encode proteins that play roles in the acquisition of nutrients during the course of infection.

HlyC, *iviVI*, and *xds* were all identified as *ivi* genes in *V. cholerae*, and are predicted to play diverse roles in the acquisition of nutrients during the course of infection. *HlyC* encodes a secreted protein that shows a high degree of similarity to the *P. aeruginosa* LipA lipase (Camilli & Mekalanos 1995). A strain with a null mutation in *hlyC* was unable to hydrolyse emulsified tributyrin thus confirming that HlyC is a secreted triacylglycerol lipase. The exact role that HlyC plays during the course of an infection has not been determined, but it is possible that this lipase is involved in the breakdown of host cell membranes to fatty-acid chains that are subsequently metabolized. An alternative possibility is that HlyC plays a role in defence against host immune cells by damaging their membranes in a way that disrupts cell function.

Xds was identified as an *ivi* gene that is induced in both the rabbit ileal loop and suckling mouse models of cholera (D. S. Merrell, M. Angelichio and A. Camilli, unpublished data). The *xds* gene encodes a secreted extracellular DNase (Focareta & Manning 1991). Though the exact role of Xds during the course of infection is not

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known, there are several possibilities. First, it may function in nutrient acquisition by breakdown of extracellular DNA into nucleotides that could then be taken up and used by *V. cholerae*. Second, Xds may degrade the DNA constituent of the mucus gel that covers the intestinal epithelium and that probably serves as a viscous barrier to attachment by the *V. cholerae* cells. Therefore, breakdown of this material by Xds would facilitate colonization. An experimental determination of the importance of Xds for virulence is complicated by the fact that *V. cholerae* produces a second, distinct extracellular DNase that may function redundantly with Xds. Focareta & Manning (1991) examined a strain in which both DNase structural genes had been disrupted, but found no significant reduction in the LD_{50} for this strain in the suckling mouse model of disease. It remains possible, however, that this double mutant strain may have a more subtle defect in colonization that is not revealed by LD_{50} determinations, but which may be revealed by a more sensitive test such as a competition assay in suckling mice.

IviVI is predicted to encode a polypeptide that has a high degree of similarity to the ATP-binding cassette (ABC) family of permeases (Camilli & Mekalanos 1995). It contains a canonical Walker A box motif that is highly conserved in members of this transporter family (Ames *et al*. 1990). ABC transporters have been well characterized in pathogens such as *S. typhimurium* and *Streptococcus pneumoniae* (Hiles *et al*. 1987; Alloing *et al*. 1990), and are often involved in the uptake of nutrients such as short peptide fragments and carbohydrates. Additionally, it has been shown that the substrate-binding components of ABC transporters are important for pathogenicity in some bacterial species in that they facilitate adhesion to host cells (Andersen 1994; Jenkinson 1992; Pei & Blaser 1993). Currently, neither the molecule that is transported by IviVI nor the role of IviVI in *V. cholerae* pathogenesis has been determined.

The initial RIVET screen for *V. cholerae ivi* genes was not comprehensive by any means, and thus it is expected that dozens, perhaps hundreds, more *ivi* genes involved in biosynthesis and nutrient acquisition await identification. Knowledge of the members of these two classes of *ivi* genes will reveal much about the nutritional status of the intestinal sites colonized by *V. cholerae*, and this body of information may apply to other pathogens that colonize the small intestine or use it as a site of entry into deeper tissues. It is possible that this information could then be used to target certain biosynthetic or nutrient uptake pathways for antimicrobial drug screening or development.

6. GENES INVOLVED IN MOTILITY

The importance of motility in the pathogenic life cycle of *V. cholerae* is a complex issue. Some data indicate that motility is required for full pathogenicity of *V. cholerae* in various adult animal models of cholera (Freter *et al*. 1981; Richardson 1991). Other data however, indicate that motility can be detrimental in the suckling mouse model. In fact, non-chemotactic mutant strains survive in greater number than wild-type vibrios and produce a more rapid and severe disease (Freter & O'Brien 1981). That motility is a detriment within the suckling mouse is supported by the following two recent findings made using RIVET. First, out of three *V. cholerae ivi* genes identified that are hypothesized to play roles in motility and chemotaxis (table 1), two of these appear to encode antisense transcripts that may be involved in inhibition of these processes. Specifically, *α-flaA* and *α-cheV* were both identified as transcriptional fusions to *tnpR* that were induced during the course of infection of suckling mice. While the biological significance of these antisense transcripts is not known, it is tempting to speculate that they are directly involved in downregulating motility and chemotaxis, respectively, during the course of infection. Second, a strain containing a null mutation in *cheV*, which encodes a putative chemotaxis protein, colonized suckling mice twice as well as the wild-type parental strain (Camilli & Mekalanos 1995). Downregulation of motility and chemotaxis could be useful for formation and maintenance of microcolonies on intestinal epithelia or, alternatively, could prevent *V. cholerae* from colonizing the wrong compartment in the small intestine: either would act as a means of increasing the efficiency of the infection. These suppositions are also supported by data that indicate that motility and virulence gene expression are reciprocally regulated in *V. cholerae* (Gardel & Mekalanos 1996; Harkey *et al*. 1994).

7. GENES OF UNKNOWN FUNCTION

The last class of *V. cholerae* genes identified by RIVET are those that are predicted to encode polypeptides with no similarities to database sequences or that are similar to hypothetical polypeptides of unknown function. This intriguing class of genes has been a major class identified by all previous IVET screens. This fact points to our still-fledgling knowledge of the roles that many gene products play in the growth and infectious process of pathogenic microbes and points to the necessity for studies designed to elucidate the roles of these many genes. Although genome sequencing projects are dramatically increasing the number of hypothetical polypeptides to which some Ivi polypeptides have striking similarity, future research must begin to focus on the elucidation of the actual functions of these many factors and their potential roles in pathogenicity.

8. THE 'NEW AND IMPROVED' RIVET

The original RIVET suffered from two limitations. First, it was somewhat laborious due to the fact that strains recovered from the host animal had to be screened via replica-plating to determine those that had resolved and were now Tc^s. Second, RIVET was exquisitely sensitive, in that only fusions that were transcribed at very low levels *in vitro* could be constructed and maintained in the unresolved state. This limited the scope of *ivi* genes that could be identified to include only those that were transcriptionally silent *in vitro*, but then were subsequently upregulated *in vivo*. This restriction excluded all of the known pathogenicity genes of *V. cholerae*, and thus, probably, some unknown genes as well. Modifications to RIVET, in the forms of a modified excisable cassette that allows selection of resolved strains and a system for

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reduced sensitivity, have dealt with each of these limitations and increased the range of studies that can now be conducted.

The most obvious way to decrease the labour required for performing RIVET screens was to modify the excisable cassette in such a manner as to allow direct selection of resolved strains after recovery from infected host tissues. This modification has been accomplished by the incorporation of a counter-selectable marker, *sacB*, into the sequence flanked by the *res1* sites. *SacB* encodes levansucrase, which is an enzyme that results in the metabolism of sucrose into a by-product that is toxic to many Gram-negative bacterial species. *V. cholerae* strains that carry the gene and are grown in the presence of sucrose are inviable (Butterton *et al*. 1993). Therefore, resolved strains, which have lost the *sacB*-containing cassette, can be directly selected for by plating small intestinal homogenates on sucrose-containing media. This facilitates screening large numbers of output cells for those containing fusions that were activated *in vivo*.

Use of the original RIVET strategy was greatly limited by the extreme sensitivity of the system. This sensitivity was a function of the fact that the produced resolvase need act at only one target sight per genome, and thus very low levels of transcription of a *tnpR* fusion would result in resolution. Recently, however, this limitation has been largely overcome by the creation of a 'tunable' RIVET whereby the sensitivity is lowered in user-defined increments (Lee *et al*. 1999). Specifically, several alleles of *tnpR* were generated that contain down-mutations in the ribosome-binding sequence (RBS). Accordingly, these alleles show a range of decreased translational efficiencies and, therefore, decreased productions of resolvase for any given level of transcription.

The mutant RBS alleles of *tnpR* were generated by polymerase chain reaction using a partially degenerate forward primer that randomized three critical bases in the RBS. These mutant alleles were transcriptionally fused to an iron-repressible promoter, P_{irgA}, within the genome of a *V. cholerae* strain containing the *res1–tet–res1* cassette. Finally, the resulting fusion strains were individually screened for those that showed decreased levels of resolution after growth at an iron concentration low enough to result in 100% resolution of the wild-type *tnpR* fusion strain. Three *tnpR* alleles were isolated that, upon further study using the P_{irgA} fusion strain, were found to require two- to fourfold lower concentrations of iron to produce 50% resolution. One of the three *tnpR* alleles was subsequently shown to serve as a very useful reporter gene for detecting the *in vivo* induction of several *V. cholerae* virulence genes (see § 9). Use of these 'reduced sensitivity' *tnpR* alleles in new RIVET screens should allow identification of a broader class of *ivi* genes: specifically, ones having low to moderate basal levels of expression during *in vitro* growth, but that are induced to yet higher levels during infection.

Use of the enhanced RIVET to screen for *ivi* genes thus proceeds by first generating several gene fusion libraries, one for each mutant RBS *tnpR* allele. For example, three libraries could be generated; one that uses the wild-type *tnpR* gene, a second that uses a mutant *tnpR* allele ($tnpR^{mut168}$) that is translated approximately twofold less efficiently, and a third that uses a *tnpR* allele ($tnpR^{mut135}$) that is translated approximately fourfold less efficiently. Each gene fusion library is pre-screened to collect unresolved strains, which are then passaged through the animal (figure 6). Finally, the bacteria are collected from infected tissue after the infection has run its course, and are plated on a sucrose-containing agar medium to select resolved strains. The sucrose-resistant strains contain *ivi* genes fused to *tnpR*, which were induced at some point during infection to mediate resolution. Each of the *tnpR* reporter alleles should result in the identification of distinct sets of *ivi* genes, but which will probably overlap to some extent. Specifically, the wild-type *tnpR* allele should identify *ivi* genes that are transcriptionally silent or nearly so during *in vitro* growth. The $tnpR^{mut168}$ allele should identify some of the same *ivi* genes as the wild-type allele, but in addition those that are transcribed at low levels *in vitro*. Finally, the $tnpR^{mut135}$ allele should identify some of the same genes as the other two alleles, but in addition those that are transcribed at still higher levels during *in vitro* growth.

Clearly, the comprehensiveness of the RIVET screen can be increased (or decreased) by altering two parameters. First, the number of unique gene fusion strains that are screened in animals can be increased, and this of course has a direct bearing on the comprehensiveness of the screen. Second, the number of different *tnpR* alleles used to construct the gene fusion libraries, as well as the breadth of their combined range of translational efficiencies, can be increased. This would have the effect of increasing the types of *ivi* genes that could be identified with respect to their levels of transcription *in vitro*. Theoretically, any gene whose level of transcription increases at some point during the infectious process can be identified using the enhanced RIVET.

9. USE OF THE ENHANCED RIVET TO MONITOR *IVI* GENE INDUCTION DURING INFECTION

As described above (see § 4 on *vieB*), RIVET has an additional application; monitoring the transcriptional induction of *ivi* genes during infection of a host animal. Initial attempts to monitor the spatio-temporal pattern of induction of known pathogenicity genes of *V. cholerae* using the original RIVET were unsuccessful due to the moderate basal levels of transcription of these genes *in vitro*. This was first demonstrated by the construction of transcriptional fusions of the wild-type *tnpR* gene to *tcpA* and *ctxA*. *TcpA* codes for the pilin subunit of the TCP, and *ctxA* codes for the enzymatic subunit of CT. The basal levels of expression of these genes resulted in immediate excision of the *res1–tet–res1* cassette from the chromosome, thus eliminating the possibility of monitoring expression patterns of either gene *in vivo* (Lee *et al*. 1999). However, it was found that when either *tcpA* or *ctxA* was fused to the $tnpR^{mut135}$ allele, which is translated fourfold less efficiently, resolution did not occur during *in vitro* growth but did occur *in vivo* (see first set of columns in figure 7*a*,*b*). In addition, growth of the fusion strains in AKI broth, which is the only known *in vitro* condition that induces *tcpA* and *ctxA* expression (Iwanga *et al*. 1986), also resulted in resolution.

The use of a mutant RBS *tnpR* allele to measure increases in the *in vivo* transcription of *tcpA* and *ctxA*

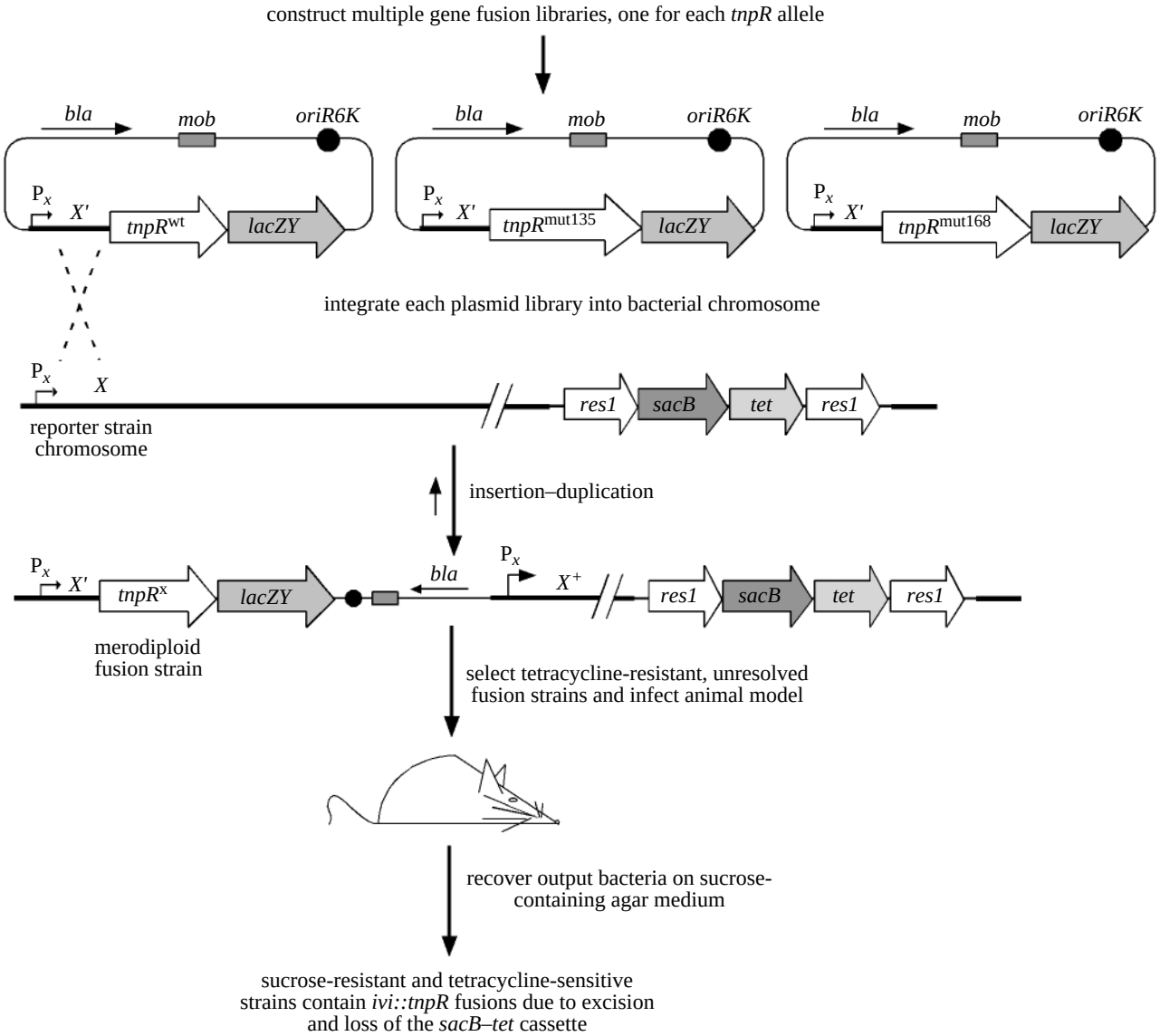

Figure 6. Graphic depiction of a RIVET screen for infection-induced genes. As shown, the screen is conducted using three different promoterless *tnpR* alleles that vary in their translational efficiencies due to different RBSs. Each *tnpR* allele will therefore yield a different class of infection-induced genes: specifically, classes of genes that differ in their basal levels of transcription during *in vitro* growth. Construction of the reporter gene fusion libraries and their subsequent screening is as in the legend to figure 1, with one notable exception: in the present scheme, resolved strains are directly selected from intestinal homogenates through their ability to grow on sucrose-containing agar media.

exemplifies what has been termed the 'tunable' RIVET. This methodology proceeds by, first, isolating a set of mutant alleles of *tnpR* that exhibit a wide range of translational efficiencies. Next, the set of *tnpR* alleles are screened for the one allele that 'tunes' the gene of interest to the appropriate level of resolvase expression, i.e. a level of expression that mediates resolution only upon an increase of gene transcription during infection. This tunable RIVET should, theoretically, make possible the analysis of any gene that shows an increase in transcription within a particular environment, such as during infection of host tissues.

The above results were the first direct experimental data confirming the long-held hypothesis that *tcpA* and *ctxA* virulence genes are transcriptionally induced during infection. The *tcpA* and *ctxA* fusions to the $tnpR^{mut135}$ allele were subsequently used to determine the effect of various regulatory gene mutations on *tcpA* and *ctxA* expression, respectively. ToxR and TcpP are homologous inner membrane proteins that sense certain environmental parameters such as pH, osmolarity and some amino acids, and in turn regulate transcription of *toxT*, *ctxA*, and other genes (Carroll *et al*. 1997; Hase & Mekalanos 1998; Skorupski & Taylor 1997). ToxR and TcpP each associate with another inner membrane protein, ToxS and TcpH, respectively, that are required for proper signalling (Carroll *et al*. 1997; DiRita & Mekalanos 1991; Hase & Mekalanos 1998). ToxT is a cytoplasmic transcription factor that is autoregulatory and, importantly, is responsible for activating transcription of a number of virulence genes such as *tcpA* and *ctxA* (DiRita *et al*. 1991). *TcpA* expression in AKI broth was found to require *toxR*, *tcpPH* and *toxT* (figure 7*a*). Interestingly though, *toxR* and *tcpPH* were not required for induction of *tcpA* during the course of a suckling mouse infection. However, mutations in all three of these regulatory genes or, a single mutation in

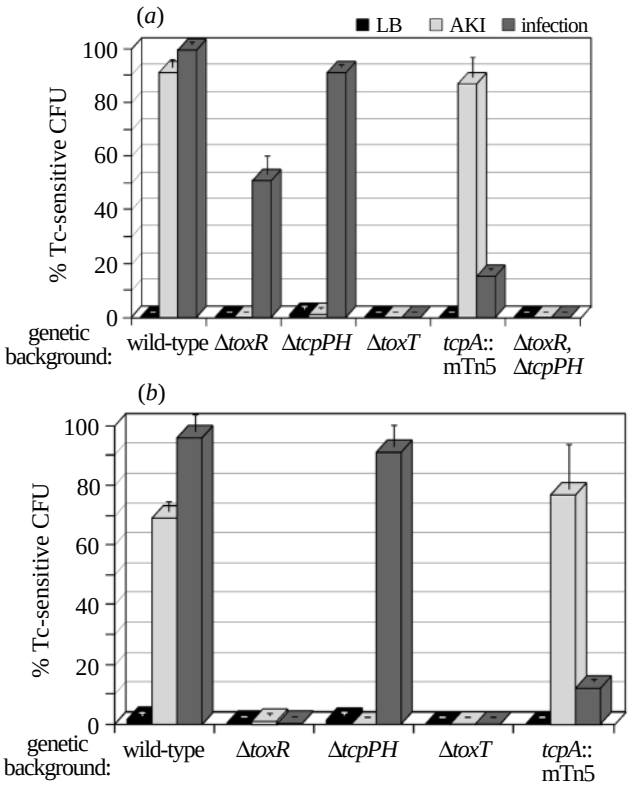

Figure 7. Regulation of *tcpA* and *ctxA* transcription during growth *in vitro* and during infection. (*a*) Extent of resolution of *V. cholerae* strains containing a *tcpA::tnpR*^{mut135} transcriptional fusion and null mutations in the genes shown on the *x*-axis after 10 h of growth in LB broth, AKI broth or in the small intestine of suckling CD-1 mice. (*b*) Extent of resolution of *V. cholerae* strains containing a *ctxA::tnpR*^{mut135} transcriptional fusion and null mutations in the genes shown on the *x*-axis. *V. cholerae* was recovered from the *in vitro* and *in vivo* environments and the per cent Tc^{s} CFUs determined. The means and standard deviations from multiple experiments are shown. Adapted from Lee *et al.* (1999).

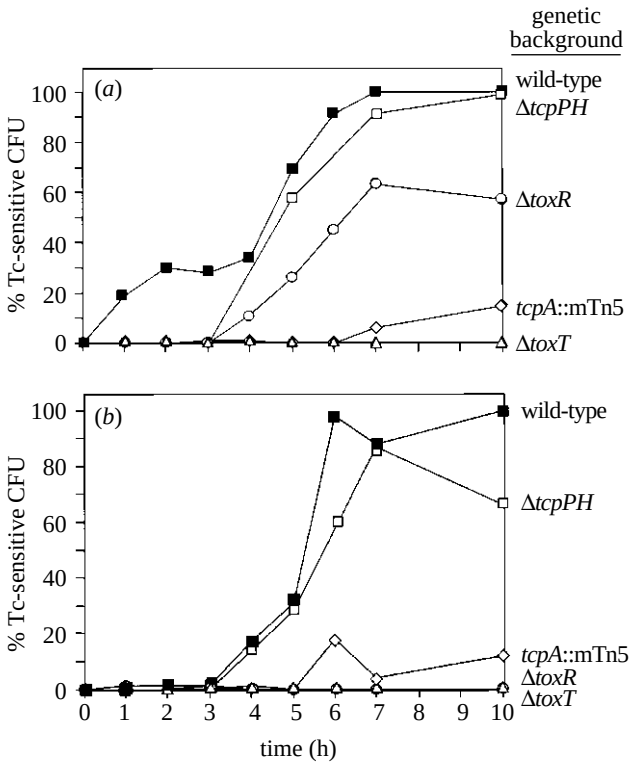

Figure 8. Kinetics of *tcpA* and *ctxA* transcriptional induction during infection. (*a*) Resolution kinetics of the same strains as in figure 7*a*, in suckling mice. (*b*) Resolution kinetics of the same strains as in figure 7*b*, in suckling mice. *V. cholerae* was recovered at the times indicated on the *x*-axis, and the per cent Tc^{s} CFUs was determined from at least 150 colonies. Genetic backgrounds are shown to the right of each curve. Adapted from Lee *et al.* (1999).

toxT, negated this induction. Hence, induction of *tcpA* during infection is accomplished in a partially redundant manner by ToxR and TcpPH, in a ToxT-dependent manner. Perhaps most surprisingly, *tcpA* transcription was found to be dependent on prior TcpA expression. This was shown by finding that a *tcpA::tnpR*^{mut135} fusion strain harbouring a null mutation in *tcpA* only resolved to low levels during infection. These data are in contrast to complete resolution of the same strain *in vitro* in AKI broth: this shows that *tcpA* autoregulation is an *in vivo*-specific phenomenon. It is likely that a TCP-mediated interaction with either the host or other vibrios, is what is required for full induction of the *tcpA* gene within the intestinal tract. However, this interaction appears to only require the low basal level of *tcpA* expression that is observed prior to, and during, the initial stage of infection (see below).

Investigation of *ctxA* expression also revealed novel aspects of the regulation of this important pathogenicity gene. Mutations in either *toxR*, *tcpPH* or *toxT*, completely abrogated transcriptional induction of *ctxA* in AKI broth (figure 7*b*). In contrast, a mutation in *tcpA* had no effect on *ctxA* expression *in vitro*. *In vivo* studies revealed that *toxR*, *toxT*, and *tcpA*, but not *tcpPH*, were each required for expression of *ctxA*. Thus, as was true for *tcpA*, there are concrete differences between the regulatory requirements for *ctxA* expression during *in vitro* growth versus during infection. Moreover, ToxR-mediated regulation of *tcpA* differs from that of *ctxA* *in vivo*, which is an unexpected result.

The above experiments provided a qualitative picture of *tcpA* and *ctxA* induction. RIVET was next used to determine the temporal patterns of expression of these virulence genes during the course of infection. Interestingly, it was found that resolution mediated by the *tcpA::tnpR*^{mut135} fusion was biphasic (figure 8*a*). Resolution starts within 1 h of infection, and levels off from 2 to 3 h. By the fourth hour, however, a further round of resolution begins, and there is a steady increase until 100% of the bacteria have resolved by 7 h. It was hypothesized that the observed resolution kinetics reflect a biphasic induction of *tcpA::tnpR*^{mut135} during infection. The first phase of induction is dependent on *toxR* and *tcpPH* as mutations in either eliminate the first, but not the second phase, of resolution (figure 8*a*). Both phases of induction required ToxT and TcpA. In contrast to this pattern of expression, *ctxA* expression does not begin until 3 h post-inoculation, and resolution (mediated by the *ctxA::tnpR*^{mut135} fusion) increases to 100% by 6 h (figure 8*b*). The induction of *ctxA* was abolished by mutations in *toxR*, *toxT* or *tcpA*, but not by a mutation in *tcpPH*. Because *ctxA* induction is dependent upon prior *tcpA* expression, it can be postulated that CT is not produced until after TCP-mediated

colonization has occurred. This latter model is gratifying from a standpoint of efficiency and potency of virulence factor expression, in that CT is not produced until after the bacteria have colonized the epithelium, which contains the target cells for CT.

Interestingly, a widely held model of virulence factor regulation in *V. cholerae* asserts that *tcpA* and *ctxA* are co-regulated (DiRita *et al.* 1991). This model is based on the coordinate regulation of genes in the ToxR-regulon, such as *tcpA* and *ctxA,* seen during *in vitro* growth. Unfortunately, due to various technical problems, it has never been possible to investigate the validity of this model *in vivo*. Use of RIVET has provided the first evidence that *in vitro* and *in vivo* regulation and expression of these two major pathogenicity factors are different. Moreover, these data represent the first indication that transcriptional induction of *tcpA* during infection is complex, wherein two phases of induction occur in response to two potentially different signals. The first signal is received soon after infection, resulting in low levels of expression of *tcpA* (Lee *et al*. 1999). Later, after colonization has occurred, we see a further increase of *tcpA* expression, presumably in response to a second, colonization-dependent signal.

All of these findings point to the extreme importance of developing an understanding of events as they occur during the course of infection in a whole animal. By using RIVET one can not only identify those genes that are induced specifically during the course of an infection, but can conduct important spatio-temporal analyses to discern the time and site of induction of each *ivi* gene. In this way, important information concerning host–pathogen interactions, such as what host compartments contain inducing signals that trigger pathogenicity gene expression, can be determined. In addition, the new 'tunable' RIVET is a powerful tool that can be used to derive an accurate picture of the regulatory hierarchy of *ivi* gene expression during infection. Specifically, the effects of mutations within regulatory and effector genes on the *in vivo* pattern of *ivi* gene expression can be easily assessed. Future use of IVET, RIVET and other *in vivo* technologies not discussed here should increase our knowledge of the physiology and pathogenicity of human pathogens, and should aid in the discovery and development of novel vaccines and treatments that will help us in our effort to control infectious diseases.

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