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MINIREVIEW

In Vivo Expression Technology

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The capacity to quickly and efficiently regulate gene expression has helped bacteria to colonize virtually every available niche in the biosphere, including dynamic and extreme ones. In accordance with this, bacterial populations must be ever ready to either take advantage of a favorable change or hunker down when the going gets tough. This proficiency is nicely demonstrated by infection of humans by facultative pathogens, for which up-regulation of genes necessary for survival and growth and down-regulation of genes deleterious to infectivity must occur on cue. To better understand this transition from ex vivo to in vivo conditions and to further our understanding of pathogenesis, it is necessary to identify genes that are specific to infection. Toward this end, in vivo expression technology (IVET) was developed (27). The purpose of this short review is to update the reader on the many variations of IVET that have been developed, to discuss nuances of each method that may be helpful to investigators embarking on studies using this technology, and to discuss offshoot technologies of IVET as tools for studying regulation of virulence genes. The many individual microbial virulence factors that have been identified using IVET are not reviewed here but have been recently reviewed by Mahan et al. (26). Because IVET is but one of several methods that can be used to screen for virulence genes induced during infection of cultured cells but is the only established method for accomplishing the same feat within infected animals, discussion herein is limited to reported uses of IVET in live animal hosts.

GETTING CLOSER TO THE ACTION

IVET was originally conceived upon the premise (now considered fact) that most virulence genes are transcriptionally induced at one or more times during infection (27). Although certain host environmental parameters can be mimicked in vitro to induce a subset of virulence genes, the full repertoire is only expressed in vivo. The beauty of IVET is that a live host, with tissue barriers and immune system intact, is used to signal induction of virulence genes. Genetic trickery, the modus operandi of IVET, is then used to identify the in vivo-induced (*ivi*) genes. As is true of all genetic screens and selections, IVET does have its limitations. The most significant of these is that the relative level and timing of transcription of an *ivi* gene largely dictates whether the gene will be identified in a particular IVET selection or screen. To date, there are four variations of IVET, and each relies on the generation of transcriptional fusions of genomic sequences to a reporter gene encoding an enzymatic activity. The variation in the four methods lies in the particular reporter gene utilized.

In the original utilization of IVET (27), advantage was taken of the fact that purine auxotrophs (in this case Δ*purA*) of *Salmonella enterica* serovar Typhimurium (referred to hereafter as *Salmonella*) are rapidly eliminated from the mouse unless they are complemented. To identify *ivi* genes, we cloned random genomic fragments directly upstream of a promoterless *purA-lacZY* synthetic operon present on a suicide vector (Fig. 1). This library was transferred by conjugation into Δ*purA* *Salmonella* and was integrated by homologous recombination to form merodiploids. A particular advantage of generating merodiploids as opposed to clean insertions (by double-crossover homologous recombination) is that *ivi* genes that are essential for survival and growth in the host have a greater likelihood of being identified. The *Salmonella* library was injected into the peritoneal cavity of mice, and systemic spread and growth in the mouse provided positive selection for strains in which an *ivi* gene was driving expression of *purA-lacZY*. Why? Because strains expressing the gene fusion in vivo became prototrophic and thrived, while strains not expressing the fusion remained auxotrophic and died. To avoid the subsequent study of strains containing constitutively active fusions, output bacteria were screened for *lacZY* expression on lactose-MacConkey indicator medium. This seminal study identified five *ivi* genes, of which three were shown to play essential roles in virulence (Table 1). Similar IVET selections, incorporating *purA* or other selectable complementing genes, have subsequently been used to identify *ivi* genes in a large number of gram-negative and gram-positive pathogens, as well as in a fungal pathogen (Table 1).

The primary advantage of the IVET selection, in comparison with other methods for identifying virulence genes en masse, lies in its simplicity: one need only generate a gene fusion library in an auxotrophic strain background and then infect a suitable host. Using positive selection in order to identify *ivi* genes makes this technique even more appealing. There are two limitations of IVET selections as follows: (i) *ivi* genes that are transiently expressed or expressed at a low level in vivo are difficult or impossible to detect because they either do not produce PurA long enough or do not produce enough of it to allow survival and growth of the strain and (ii) not all *ivi* genes are essential for infectivity. The first of these limitations is of unknown magnitude; however, it is reasonable to predict that only a subset of virulence genes that are transcrip-

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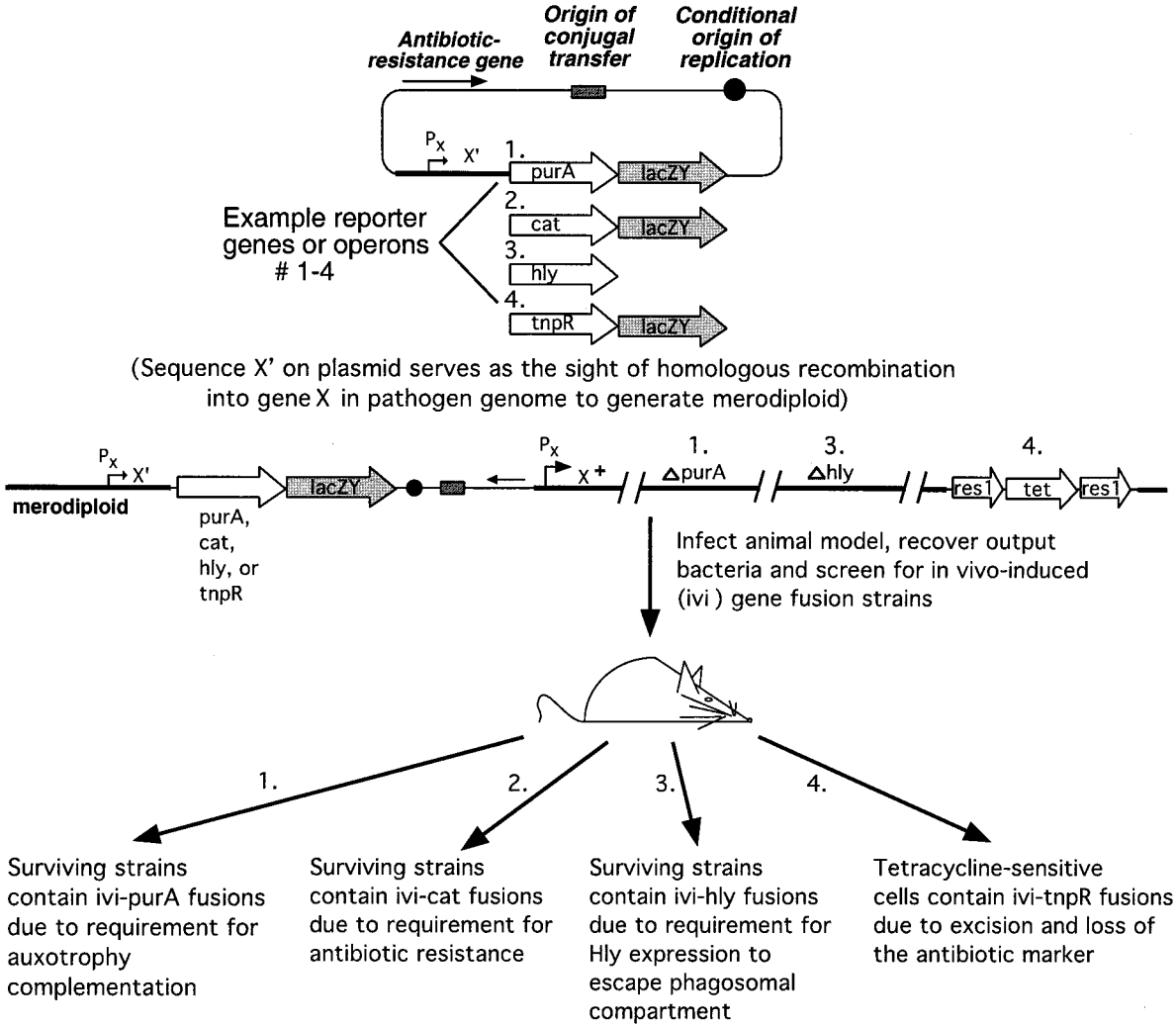

FIG. 1. Graphic depiction of four variations of IVET. Auxotrophy complementation-based selections are conducted using fusions to a promoterless *purA* or other such gene (plasmid 1), antibiotic selections are conducted using fusions to a promoterless antibiotic gene such as *cat* (reporter 2), dual reporter selections can use reporter genes such as *hly* (reporter 3) or other types of genes that provide an in vivo selection and an in vitro screen for promoter activity, and finally, RIVET screening is done using a promoterless *tnpR* gene (reporter 4), whose protein product will excise a substrate cassette (*res1-tet-res1* in figure) from elsewhere in the bacterial genome. Reporter gene fusion libraries are constructed by ligating random genomic fragments (designated 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 harboring gene fusions that are active in vitro: this is accomplished by selecting for tetracycline-resistant, $LacZ^-$ colonies. In all cases, fusion strains are passaged through an appropriate animal model of disease and collected from infected tissues or fluids after a period of time. In the case of the antibiotic-based IVET, the antibiotic (in this example, chloramphenicol) 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. Alternatively, *L. monocytogenes* strains containing infection-induced gene fusions to *hly* are selected in the host (see text for details) and are subsequently screened for lack of in vitro expression on blood agar plates (Hly will lyse the red blood cells, forming a zone of clearing around colonies). Finally, infection-induced gene fusions to *tnpR* are screened for at a postinfection stage by virtue of their tetracycline sensitivity and lack of expression of LacZ on indicator plates.

tionally silent in vitro will be expressed at levels sufficient for survival throughout the course of an infection. The second limitation, applicable to all IVET selections and screens, is often misconstrued, particularly since the advent of signature-tagged mutagenesis, which is a genetic screen used to identify genes that are essential for infectivity (16). While it is true that many *ivi* genes, when mutated singly, do not reduce infectivity in animal models, it is incorrect to conclude that such genes therefore play no role in virulence. It is becoming appreciated more and more that many virulence factors act in a partially or fully redundant manner (e.g., see references 3, 4, 15, 29, and 38) and thus mutation of one such gene is unlikely to attenuate virulence. Indeed, a fuller understanding of redundant genes involved in survival and growth in vivo can be obtained by observing those occasions when the suicide vector contains, not a promoter region, but instead an internal segment of an *ivi* gene (or operon) which upon insertion inactivates the gene (or downstream genes in an operon) in which it resides. By iden-

TABLE 1. IVET selections and screens used to identify pathogen genes induced during infection[a]

Pathogen	Host (model)	Reporter (selection or screen)	No. of *ivi* genes identified (no. essential for virulence/no. tested)[b]	Reference
Gram-negative bacteria				
Actinobacillus pleuropneumoniae	Pig (pneumonia)	*ribBAH* (selection)	10 (ND)	7
Escherichia coli	Mouse (septicemia)	*cat* (selection)	95 (5/7)	19
Klebsiella pneumoniae	Mouse (systemic infection)	*galU* (selection)	20 (ND)	21
Pasteurella multocida	Mouse (systemic infection)	*kan* (selection)	16 (ND)	18
Porphyromonas gingivalis	Mouse (abscess)	*tetA(Q)2* (selection)	14 (1/3)	41
Pseudomonas aeruginosa	Mouse (systemic infection)	*purA* (selection)	8 (ND)	11
	Rat (chronic pulmonary infection)	*purA* (selection)	8 (ND)	11
	Neutropenic mouse (systemic infection)	*purEK* (selection)	22 (1/1)	40
Pseudomonas fluorescens	Sugar beet seedling (rhizosphere colonization)	*panB* (selection)	20 (ND)	34
Pseudomonas putida	Fungal hyphae (colonization)	*pyrB* (selection)	5 (ND)	24
Rhizobium meliloti	Alfalfa (symbiosis)	*bacA* (selection)		31
Salmonella[c]	Mouse (systemic infection)	*purA* (selection)	5 (3/3)	27
	Mouse (systemic infection)	*cat* (selection)	1 (ND)	28
	Mouse (systemic infection)	*purA* (selection)	10 (0/2)[d]	12
	Mouse (systemic infection)	*cat* (selection)	6 (0/4)[d]	12
Vibrio cholerae	Infant mouse (cholera)	*tnpR* (screen)	13 (1/13)	5
	Rabbit (cholera)	*tnpR* (screen)	1 (0/1)	30
Yersinia enterocolitica	Mouse (systemic infection)	*cat* (selection)	22 (1/1)	10
	Mouse (Peyer's patch colonization)	*cat* (selection)	48 (4/4)	42
Xanthomonas campestris pv. campestris	Turnip seedling (rot)	*cat* (selection)	14 (ND)	32
Gram-positive bacteria				
Listeria monocytogenes	Mouse (systemic infection)	*hly* (selection)	9 (1/1)	8
Staphylococcus aureus	Mouse (renal abscess)	*tnpR* (screen)	16 (7/11)	25
Streptococcus gordonii	Rabbit (endocarditis)	*cat* (selection)	13 (ND)	20
Fungi				
Histoplasma capsulatum	Mouse (systemic infection)	*ura5* (selection)	20 (ND)	36

[a] IVET selections or screens done using other types of models, such as cultured mammalian cells, are not listed (see text for an explanation).
[b] In some reports, both animals and cultured cells were used as hosts. In these cases, only those in vivo-induced (*ivi*) genes identified in animal hosts are listed. Only genes whose identities were reported are listed here. *ivi* genes identical or homologous to previously known virulence genes, but which were not tested for roles in virulence, are not included. ND, not done; indicates that no *ivi* genes were tested for possible roles in virulence in the study.
[c] *Salmonella* refers to *S. enterica* serovar Typhimurium.
[d] In this report, possible mild defects in virulence were not tested for, i.e., only major defects would have been noted.

tifying such genes, the experimenter has not only identified an in vivo-induced gene but has learned that the *ivi* gene is not coding for an essential function by itself, and a search for possible redundant factors can then be pursued.

With IVET, as with other techniques that are used to search for virulence genes, the animal model being used often limits the "search light" to particular stages of the infectious life cycle of a pathogen and therefore a particular *ivi* gene may serve a role in a stage not being examined. For example, one stage that is rarely investigated is transmission of an infectious agent, which, along with multiplication in the host, is of supreme importance to any professional pathogen. To illustrate this point, cholera toxin (an Ivi protein) is not necessary for *Vibrio cholerae* to adhere to and multiply within the infant mouse small intestine (a widely used animal model to study this pathogen), and yet this potent toxin is clearly a major pathogenicity factor in human disease and in transmission of this waterborne agent back into aqueous environments (17, 33). Perhaps, where possible, we should begin to look at pathogens in their naturally occurring environment to better understand the entire pathogenic lifestyle (31).

A second variation of IVET involves the use of antibiotic resistance genes as selectable reporters (28) (Fig. 1). By this strategy, treatment of the infected host with the appropriate antibiotic selects for bacterial strains harboring active gene fusions. This technique was used by Heithoff et al. (12) to identify more than 100 *ivi* genes in *Salmonella*, though the identities of most of these genes have not yet been reported. This type of IVET approach was actually used prior to the inception of the term "in vivo expression technology" to select *Xanthomonas campestris* pv. *campestris* genes induced during infection of a plant host (32). This antibiotic-based method has subsequently been applied to a number of other pathogens (Table 1). The primary advantage of this IVET strategy lies in the fact that complementable auxotrophy in the strain of study is not needed. However, this strategy still requires that the pathogen be transformable and exhibit either (i) homologous recombination for generating chromosomal fusions to IVET reporter genes or (ii) plasmid maintenance for generating promoter fusions to IVET reporters on an episome. A disadvantage of this approach is that the antibiotic must be administered to the host animal and must penetrate to the site of

infection. This requirement, in turn, provides some flexibility to the IVET selection in that the antibiotic can be given at lower doses or at specific times of infection in order to increase the breadth of *ivi* genes identified. The latter was nicely demonstrated by the differential selection for populations of *ivi* genes that were induced during different stages of *Yersinia enterocolitica* infection (10, 42) (Table 1). Administration of the antibiotic at an early stage of infection, soon after intragastric inoculation, allowed the investigators to identify *Y. enterocolitica ivi* genes induced during colonization of the Peyer's patches (42), and in a separate study, the antibiotic was administered at a later stage to intraperitoneally infected mice to identify *ivi* genes induced during systemic infection in the liver and spleen (10). Of the genes identified, only the siderophore receptor *fyuA* was found in both screens. These two implementations of IVET exemplify the ability to inventory genes necessary for different sites and stages of colonization and the ability of IVET to tease out tissue-specific virulence factors.

A third type of IVET selection uses a single gene as a dual reporter, providing for both in vivo selection of active gene fusions and later screening of fusions that are transcriptionally silent during in vitro growth. The first such dual reporter used was *hly*, encoding the pore-forming hemolysin listeriolysin O (LLO) of *Listeria monocytogenes* (8). LLO mediates lysis of the phagosomal membrane in macrophages and in other cell types that have engulfed *L. monocytogenes* (39). This reporter provides an in vivo selection for active fusions that allow for escape from the phagosomal compartment and subsequent multiplication of *L. monocytogenes* in the cytoplasm as well as a convenient screen for inactive fusions on blood agar plates in vitro (such colonies show no hemolysis). Because expression of the reporter is required at the stage of phagosomal containment, *ivi* genes that are expressed in the phagosomal environment are identified. Another dual reporter is *galU* from *Klebsiella pneumoniae* (21). GalU is required for lipopolysaccharide and capsule synthesis, which, in turn, is required for survival in vivo (6). GalU also allows for a convenient plate screen of failure to ferment galactose on MacConkey agar to identify fusions that are transcriptionally silent in vitro (6). The use of a dual reporter gene simplifies the design of an IVET vector and, in the case of *hly*, provides unique specificity to the class of *ivi* genes identified.

Recombination-based IVET (RIVET) is the fourth IVET strategy for identifying *ivi* genes and the only one developed so far that functions as a genetic screen. In this case, fusions are made to a promoterless resolvase gene such as *tnpR* from Tnγδ (35). Prior to this step, a gene cassette that serves as the substrate for resolvase is placed at a neutral site in the bacterial genome. Typically, the substrate is an antibiotic resistance gene flanked by resolvase recognition sequences. An *ivi* gene fused to *tnpR* results in resolvase production, whose action results in the permanent excision of the antibiotic marker (a reaction termed resolution). This event marks the bacterium by endowing it with an inheritable antibiotic-sensitive phenotype. Resolved strains are then screened for (by replica plating of colonies) after recovery of the bacteria from infected tissues.

The RIVET method has distinct advantages and disadvantages relative to the other IVET approaches. Because only a small pulse of resolvase expression is needed to mediate resolution, the method is exquisitely sensitive to low or transient expression of the *ivi* gene during infection and is therefore capable of identifying these potentially interesting classes of genes. This sensitivity is a double-edged sword, though, in that *ivi* genes with low to moderate basal levels of expression in vitro cannot be identified because such genes result in immediate resolution during strain construction. In the absence of taking steps to reduce the sensitivity of the system (23), the number of *ivi* genes that can be identified in any particular pathogen is likely to be restricted. A second advantage of RIVET is that no selective pressure is placed upon the bacteria during infection, which is not true for IVET selections, and thus the infection is guaranteed to proceed on a natural course. Finally, use of RIVET to study induction of virulence genes in vivo is limited in two other ways: first, only the initial induction of an *ivi* gene can be assayed, since resolution is irreversible, and thus expression at later times or within downstream host tissues cannot be detected; and second, no quantitative information concerning gene expression levels is provided. Because of these and other unique features (described below), the acronym RIVET is often used to distinguish it from the more commonly used IVET selection methods. RIVET has been used to identify *ivi* genes in *V. cholerae* and with greater success in *Staphylococcus aureus* (5, 25).

OFFSHOOT TECHNOLOGIES TO STUDY THE REGULATION OF *IVI* GENES

Because *ivi* genes, by definition, are transcriptionally silent during in vitro growth and induced during infection, it is difficult to study their regulation using standard methods. Several approaches have been developed, however, which circumvent or even take advantage of this limitation in order to study the regulation of *ivi* genes.

Examining the spatial and temporal patterns of induction of *ivi* genes. An interesting alternative use of RIVET is the monitoring of induction of transcription of *ivi* genes as a function of time and location in the host. To do this, a pathogen (containing an *ivi-tnpR* fusion) is isolated at different times during infection or from specific tissues, and then the recovered bacteria are assayed to determine the percentage that have resolved. This technique was utilized by Lee et al. (23) to study the induction patterns of several virulence genes within wild-type and mutant strains of *V. cholerae* during infection. Induction of two major virulence genes that were thought to be coinduced based on in vitro studies were found to be induced in a sequential manner in the infant mouse small intestine. Moreover, it was shown that induction of the first of these was needed in order for the second to be expressed. These results provide tantalizing clues to what appears to be a highly coordinated and host-pathogen interaction-dependent program of virulence gene expression by *V. cholerae*.

In addition to these findings, Lee et al. (22) used RIVET to assess the roles of upstream regulatory factors for induction of these virulence genes. To accomplish this, individual genes encoding known regulators were mutated in an *ivi-tnpR* fusion strain background. Next, the mutant strains were inoculated into animals and the temporal patterns of induction were determined. If the pattern of induction is unchanged from the parent strain, then the regulator tested plays no essential role in regulating the *ivi* gene in vivo. On the other hand, any

change in the temporal pattern of resolution, such as loss of induction altogether, reflects a role for the regulator in regulating the virulence gene during infection. Once again, it was shown that the insights gained from in vitro studies were not borne out in vivo; Lee et al. found that there were differences in the requirements for particular virulence regulators in vivo versus in vitro. For example, it was found that the transcriptional regulator TcpP and its accessory protein TcpH, although required for cholera toxin gene expression in vitro, are not required for expression during infection in the infant mouse model of cholera.

As mentioned earlier, because resolution is irreversible, spatio-temporal experiments using RIVET are limited to assaying the initial induction patterns. For these reasons, quantitative reverse transcriptase PCR is currently the method of choice for quantitative spatio-temporal studies of in vivo gene expression, at least for experimental systems in which sufficient numbers of bacterial cells for mRNA isolation are present in host tissues (9, 37). An alternative method for spatio-temporal studies is the use of a light-emitting reporter, such as Gfp, to detect gene expression (1). Use of Gfp is limited in some cases by background fluorescing particles which interfere with readings from bacteria recovered from infected host tissues (1).

Identifying transcriptional regulators of *ivi* genes. Because *ivi* genes are transcriptionally silent during in vitro growth, it is possible, in some cases, to screen or select for mutations in loci encoding regulators of the *ivi* genes. For example, consider the generic case of a strain containing a fusion of an *ivi* gene to *purA-lacZY*: selection for strains with mutations in a repressor of *ivi* can be carried out simply by demanding growth on media lacking purines. Alternatively, one could screen for mutant strains that form blue colonies on agar plates containing X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). Heithoff et al. (14) used the latter method to identify a semiglobal repressor of *Salmonella ivi* genes. The repressor turned out to be Dam (DNA adenine methylase). A null mutation in *dam* resulted in derepression of ~20% of the previously identified *ivi* genes in *Salmonella* and also attenuated virulence substantially. Because a *dam* strain both expresses *ivi* genes inappropriately and is avirulent, such strains have potential as live or killed vaccine strains (13, 14).

Recently, Lee et al. (22) developed a genetic selection to identify positive regulators of *ivi* genes. This method takes advantage of a property of RIVET, excision of the antibiotic resistance marker in vivo, to select for mutant strains that instead retain the antibiotic marker after animal passage. Such strains contain mutations in positive regulators of the particular *ivi* gene. A unique aspect of this selection method is that the regulators identified must function during infection. This is likely to be a useful genetic tool for probing regulatory networks that are active during infections but which may not be active during growth in vitro. In the above study, a number of positive regulators of the cholera toxin genes (*ctxAB*) were identified, and these included genes involved in chemotaxis and other signal transduction pathways. These signaling pathways were not required for *ctxAB* induction in vitro during growth under specialized conditions that induce *ctxAB* (22).

CONCLUDING REMARKS

The coordinated regulation of bacterial virulence factors is critical to successful infection. This requires that a set of genes be up-regulated during infection while, concurrently, another set is down-regulated. If a pathogen has in effect been keeping a particular set of genes in reserve for the appropriate moment in host tissues, then it is likely that these genes play some role in virulence: it is up to us to figure out what these specific roles are. Understanding the timing, tissue specificity, and regulation of an *ivi* gene can set the stage for additional studies directed at deciphering the precise role of the encoded protein.

Here we have given a description of the various forms of IVET and the major advantages and disadvantages of each. While each IVET approach has been shown to be limited in one way or another, the importance of this technology is indisputable. Many reports listed in Table 1 have shown the requirement of one or more *ivi* genes for infection of a host, and many more bona fide virulence genes have been identified through IVET screens and selections done using cultured cells or using other in vitro models of infections that were not discussed here. Indeed, 9 years after the advent of IVET, the technology has become more than a bellwether (2)—it has become a utility for virulence gene discovery in many pathogens and a stimulus for the creation of new tools to investigate pathogenicity.

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