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In Vivo Expression Technology (IVET) and Its Application in Plant-Associated Bacteria

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***In vivo* expression technology (IVET) has been developed to study bacterial gene expression in *Salmonella typhimurium* during host infection. The expression of selected genes by IVET has been elevated *in vivo* but not *in vitro*. The selected genes turned out to be important for bacterial virulence and/or pathogenicity. IVET depends on a synthetic operon with a promoterless transcriptional fusion between a selection marker gene and a reporter gene. The IVET approach has been successfully adapted in other bacterial pathogens and plant-associated bacteria using different selection markers. *Pseudomonas putida* suppresses citrus root rot caused by *Phytophthora parasitica* and enhances citrus seedling growth. The IVET strategy was adapted based on a transcriptional fusion, *pyrBC'-lacZ*, in *P. putida* to study the bacterial traits important for biocontrol activities. Several genes appeared to be induced on *P. parasitica* hyphae and were found to be related with metabolism and regulation of gene expression. It is likely that the biocontrol strain took a metabolic advantage from the plant pathogenic fungus and then suppressed citrus root rot effectively. The result was parallel with those from the adaptation of IVET in *P. fluorescens*, a plant growth promoting rhizobacteria (PGPR). Interestingly, genes encoding components for type III secretion system have been identified as rhizosphere-induced genes in the PGPR strain. The type III secretion system may play a certain role during interaction with its counterpart plants. Application of IVET has been demonstrated in a wide range of bacteria. It is an important strategy to genetically understand complicated bacterial traits in the environment.**

Keywords : biocontrol, IVET, PGPR, *Pseudomonas putida*, type III secretion.

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In Vivo Expression Technology (IVET)

The recent adaptation of transcriptional gene fusion has greatly facilitated the study of bacterial gene expression (Slauch and Silhavy, 1991). A new genetic approach, termed *in vivo* expression technology (IVET), has been developed in animal bacterial systems based on gene fusion to identify bacterial genes specifically induced during host infection (Mahan et al., 1993b). The IVET used the animal as a selective medium to isolate bacterial genes. The genes identified by IVET were poorly expressed on laboratory media but showed elevated level of expression in host tissue. IVET depends on a synthetic operon, a promoterless transcriptional fusion between a selection marker gene and a reporter gene. The *in vivo*-induced (*ivi*) genes can be identified by their ability to express a promoterless selection marker gene that is essential for survival*in vivo*. Expression *in vitro* can then be monitored by studying the expression of another promoterless reporter gene located downstream of the selection marker (Fig. 1). The IVET strategy, technical aspects, and its modification with recombinase-based approach (RIVET) were recently reviewed in detail for broad application of the approach in animal bacterial systems (Slauch and Camilli, 2000).

The IVET strategy has advantages over traditional mutagenesis techniques since there is a positive selection for genes that are specifically induced by environmental cues *in vivo*. The procedure does not disrupt genes, which may be essential *in vivo*, by integrating the fusion into target bacterial chromosome through homologous recombination (Mahan et al., 1993b). The IVET system then takes an advantage from transduction using bacteriophage to clone *ivi* genes (Mahan et al., 1993a). Recently, plasmid-based IVET strategies have also been used successfully (Lee and Cooksey, 2000; Young and Miller, 1997).

Numerous *ivi* genes have been identified through the IVET approach from a number of animal pathogenic

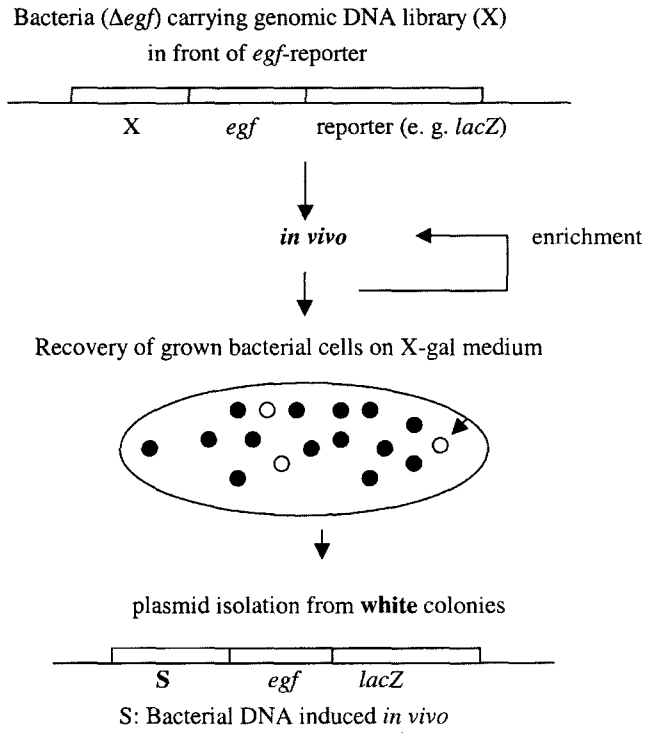

Fig. 1. *In vivo* selection procedure to isolate clones of target bacteria induced *in vivo*. A genomic DNA library is constructed in front of a promoterless transcriptional fusion between an essential growth factor (*egf*) gene and a reporter gene (e.g. *lacZ*). X represents the random DNA fragment isolated from the wild type bacteria. Pools of the *egf* mutant bacteria carrying genomic DNA library are applied *in vivo*. Bacterial cells grown or survived *in vivo* can be recovered and plated on media to monitor the reporter gene expression *in vitro*. When *lacZ* is used as a reporter gene, white cells will be selected to isolate plasmids. Each plasmid carries a DNA fragment of target bacteria induced *in vivo*. S represents the gene induced *in vivo*.

bacteria. Mutations on *ivi* genes affect the virulence of the pathogenic bacteria, indicating that the identified *ivi* genes are important in infection and/or pathogenicity (Camilli and Mekalanos, 1995; Lowe et al., 1998; Mahan et al., 1993b; Mahan et al., 1995; Wang et al., 1996). The identified *ivi* genes from animal pathogen fall into the following categories: (1) regulatory genes, (2) RpoS-regulated genes, (3) genes for metabolic functions, (4) adhesin- and invasin-like genes, and (5) unknown genes (Heithoff et al., 1997). The IVET selection strategy in *Salmonella typhimurium* identified a number of previously uncharacterized genes in addition to genes encoding previously described virulence factors. Those uncharacterized genes turned out to be important in bacterial virulence through mutagenesis studies (Heithoff et al., 1997; Mahan et al., 1993b). Interestingly, majority of the *ivi*- selected genes often do not match with any genes in the databases, and in some case they are more than 50% of the total identified *ivi* genes. The *ivi* genes from animal pathogens provided a unique opportunity to develop a new attenuated live vaccine or to define a new drug target (Heithoff et al., 1999). One of the important outcomes from IVET is detection of Dam methylase in *S. typhimurium*, which is a key regulator for virulence expression in the pathogen (Mahan and Low, 2001). This IVET approach will make it possible to identify the genes induced by different environmental signals. Therefore, the IVET strategy has a great potential in the study of bacterial gene expression *in vivo* and in dissecting bacterial traits in various environments.

Other strategies to identify *in vivo*-induced genes

Subtractive hybridization is another way to identify genes differentially expressed, but it has limitation in a bacterial system because bacterial mRNAs have a short half-life. The technology of microarrays of immobilized DNA or oligonucleotides (DNA chips) was recently developed (Ramsay, 1998; Schena et al., 1995). The chip technology has potential for screening differentially expressed bacterial genes (de Saizieu et al., 1998). However, the application of the DNA chip technology is still limited because of high cost and short half-life of mRNAs from bacteria isolated in many environments. A powerful alternative strategy to identify bacterial genes induced during host infection, termed signature-tagged mutagenesis (STM), has been also developed (Hensel et al., 1995). The STM based on transposon mutagenesis could also be used to identify bacterial genes differentially expressed *in vivo* (Hensel et al., 1998). While IVET takes advantage of positive selection to identify *in vivo*-induced genes, STM is based on a negative selection that requires additional screening process. However, STM is still one of popular strategies in identifying bacterial virulence genes and has been applied in many animal bacterial systems (Autret et al., 2001; Polesky et al., 2001; Shea et al., 2000).

Selection markers for IVET

The choice of selection marker is crucial in the application of IVET in a different bacterial system. In the original IVET approach for *S. typhimurium*, a purine requirement for bacterial survival was used as the selection criterion during bacterial infection of the host (Mahan et al., 1993b). The IVET system used a *purA*-disrupted mutant *S. typhimurium*, which is deficient in purine biosynthesis. The *purA* gene supplied *in trans* by IVET construct was a selection marker. Similarly, other genes required for cell essential component biosynthesis can be utilized as a selection marker by the same principle (Handfield et al., 1998). It is also evident that classical antibiotic resistance

Table 1. IVET and selections in various bacteria

Bacteria	Selection marker/reporter	Reference
Salmonella typhimurium	*purA-lacZY*	Mahan et al., 1993b
Salmonella typhimurium	*cat-lacZY*	Mahan et al., 1995
Vibrio cholerae	*tnpR* (resolvase)	Camilli and Mekalanos, 1995
Pseudomonas aeruginosa	*purEK*	Wang et al., 1996
Staphylococcus aureus	*tnpR*	Lowe et al., 1998
Klebsiella pneumoniae	*galU*	Lai et al., 2001
Pseudomonas fluorescens	*panB-lacZY*	Rainey, 1999
Pseudomonas putida	*pyrBC-lacZ*	Lee and Cooksey, 2000

genes are feasible as selection markers, and that chloramphenicol acetyltransferase (*cat*) gene has been successfully applied in IVET (Mahan 1995; Young and Miller, 1997). Other essential genes for bacterial growth and/or survival *in vivo* were also used as selection markers to apply IVET system during various bacterial infections of animal cells (Camilli and Mekalanos, 1995; Handfield et al., 1998; Lowe et al., 1998; Wang et al., 1996).

To adapt the IVET system in environmental microbiology including plant-associated bacterial system, several appropriate selection systems are possible. Antibiotic resistance marker gene is not suitable in many bacteria as a selection marker since the antibiotics as selection have some limitations. Natural environments already exhibit diverse microbial populations including antibiotics resistant microorganisms. In addition, some antibiotics are not effective because of their inactivation by being absorbed by physical matters or degraded either chemically or biologically. Early studies showed that *thyA*, involved in thymidine biosynthesis, could be successfully used as an alternative autoselective marker (Ross et al., 1990; OFlaherty et al., 1995). Applications of IVET or its variations carried out with different selection systems in animal bacteria and in plant-associated bacteria are summarized in Table 1.

Application of IVET in the biocontrol bacterium *Pseudomonas putida*

Rhizobacteria, such as fluorescent pseudomonads and *Bacillus* spp., have received much attention as an alternative way to control soil-borne diseases because of their potential usage and growing public concerns over pesticides (Cook 1993). Disease suppresiveness and plant growth promotion by soil-borne microorganisms have been exploited and microorganisms have been applied as biocontrol agents (Weller, 1988). A group of potential biocontrol organisms is the fluorescent pseudomonads that suppress a wide range of root diseases. The biocontrol mechanisms and traits of fluorescent pseudomonads vary (Handelsman and Stabb, 1996). *P. putida* strain 06909 was isolated from citrus rhizosphere as a contaminant of *Phytophthora parasitica* causing citrus root rot (Turney, 1995). Greenhouse and field experiments showed that this particular strain enhances citrus seedling growth and protects young citrus trees from *P. parasitica* (Steddom and Menge, 1999). Biocontrol activity of *P. putida* 06909 depends on two bacterial traits, siderophore production and fungal hyphae colonization, which mimics bacterial parasitism of *Phytophthora* hyphae. It has been shown previously that bacterial flagella are responsible for the bacterial attachment on fungal hyphae (Yang et al., 1994). However, interaction between *P. putida* 06909 and *P. parasitica* is not well understood. IVET was adapted to genetically dissect the interaction between *P. putida* 06909 and *P. parasitica* (Fig. 1). The rationale to adapt the IVET strategy is based on the hypothesis that genes specifically induced during fungal colonization are important in the interaction between fungus and bacteria and may contribute to improved biocontrol activity of *P. putida* 06909.

The IVET was based on the promoterless transcriptional fusion between *pyrBC'* and *lacZ* residing in a broad host range plasmid, pRK415. *pyrBC'* encode a dodecameric complex enzyme, aspartate transcarbamoylase (ATCase), which is a key enzyme for pyrimidine biosynthesis (Schurr et al., 1995). Experiments with *pyrB* mutant of *P. putida* 06909 indicated that *pyrBC'* could be a good selection marker for the application of IVET to study the bacterial colonization of fungal hyphae and citrus rhizosphere (Lee et al., 1998). Initial screening isolated about 30 clones specifically induced during fungal hyphae colonization. Nineteen of these clones were also induced during late growth phase, which may be important for bacterial survival at stationary phase during fungal hyphae colonization. They are not fully characterized but could be regulated by RpoS, similar to the case of RpoS regulated genes in animal bacteria. Eleven clones included some siblings and it turned out that five of them are unique as genes specifically induced during fungal hyphae colonization. Following are

the potential gene products: diacylglycerol kinase and LuxR family response regulator, ATP binding cassette (ABC) transporter and LysR family response regulator, substrate specific outer membrane porin, sodium/proton antiporter like membrane protein, and an unknown protein (Lee and Cooksey 2000). A mutant on ABC transporter and LysR family response regulator somewhat impaired fungal growth inhibition while the mutant still retained the fungal hyphae colonization (Unpublished). It was suggested that the ABC transporter might be involved in either secretion or uptake of specific chemicals from fungal hyphae. The isolation of candidate chemical for ABC transporter was not successful. The role of a LysR family response regulator in the upstream of ABC transporter is not clear. It may be involved in the regulation of the ABC transporter gene expression or in the regulation of cadmium resistant determinant located in the right upstream (Lee et al., 2001). Mutation on the other loci should be constructed and tested for the involvement of each gene for the bacterial-fungal interaction. Second screening has isolated additional unique clones and studies to define the role of isolated genes during interaction with fungus are in progress. It is likely that majority of the induced genes during fungal hyphae colonization are involved in bacterial metabolism, regulation of bacterial gene expression, and bacterial survival.

Application of IVET in other plant-associated bacteria

IVET system has also been applied in other plant-associated bacteria including bacterial plant pathogens, epiphytic bacteria, and plant growth promoting rhizobacteria (PGPR). Most of them are not published except one study with PGPR strain *P. fluorescens* (Rainey, 1999; Preston et al., 2001). A number of unique selection markers have been used for the modification of IVET. The first application of IVET in environmental microbiology was done in a PGPR strain, *P. fluorescens* SBW25, to isolate genes showing elevated levels of expression in the rhizosphere (Rainey, 1999). An essential gene, *panB*, for pantothenate biosynthesis was successfully used in the IVET approach. The isolated genes were similar to the genes encoding metabolic functions, regulatory functions, and some unknowns. These results were parallel with those from the biocontrol strain *P. putida* 06909. One of the interesting genes isolated was a homologue of type III secretion system, and consequent study has isolated an entire type III gene cluster from the *P. fluorescens* strain (Preston et al., 2001). The *P. fluorescens* did not elicit hypersensitive response (HR) from any of the host plants tested but elicited an AvrB-dependent HR in *Arabidopsis thaliana*. In addition, the type III gene cluster was widely distributed among many PGPR strains, suggesting that the type III system of the PGPR strain may play an important role in rhizosphere. The ecological significance of the rhizosphere induced type III genes of *P. fluorescence* is not understood.

The application of IVET is also in progress in a number of plant epiphyte and plant pathogenic bacteria. Genes induced during epiphytic survival and growth in *P. syringae* pv. *syringae* were isolated by adapting IVET system with methionine biosynthesis genes, *metXW*, as a selection marker (Andersen et al., 1998). Similarly, genes induced during plant infection were identified by IVET approach in plant pathogenic *P. syringae* pv. *tomato*. The IVET used in *P. syringae* pv. *tomato* was based on the promoterless transcriptional fusion between *hrcC* and *gus*. These two approaches have also identified type III secretion system genes to be induced during interaction with host plants. The latter study mostly identified virulence factors, avirulence genes, stress related protein genes, and several genes encoding unknown functions (Unpublished). Both studies have shown that IVET is a good system to study bacterial gene expression in many plant-associated bacteria

Future directions

The development of IVET in animal bacterial systems has led to the potential to produce live vaccines and to identify new drug targets. Dam methylase identified in *S. typhimurium* by IVET turned out to be a key regulator for virulence of the pathogen, and that *dam*-mutant might be a good vaccine (Heithoff et al., 1999). In addition, *dam* mutated *Erwinia chrysanthemi* failed to cause disease in African violets although the mutant still produced pectic enzymes (Mahan and Low, 2001). Therefore, there is still a possibility to find out novel genes important for virulence and/or pathogenicity by adapting IVET in plant pathogenic bacteria. The application of IVET is not limited to pathogenic bacteria. A handful of studies showed that the strategy could be applied in environmental microbiology including plant-associated bacteria. Several useful selection markers have already been developed and the use of the selection marker should be feasible in other bacteria. The adaptation of IVET in other bacterial system would facilitate the understanding of bacterial traits in various natural environments.

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