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Unraveling the Secret Lives of Bacteria: Use of In Vivo Expression Technology and Differential Fluorescence Induction Promoter Traps as Tools for Exploring Niche-Specific Gene Expression

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INTRODUCTION

The overwhelming focus for microbiology during the last century has been the study of microbes under well-defined laboratory conditions. The value of this approach is evident in the wealth of information now available on physiological and genetic mechanisms, without which the rapid advances in molecular microbiology would not have been possible.

The utility of studying bacteria in vitro remains clear, particularly in light of technologies for genome-scale analysis in conjunction with the ability to carefully control biotic and abiotic environmental factors in the laboratory (184, 199, 238). For example, virulence factors of animal pathogens have been identified by analyzing bacterial responses to changes in temperature (166, 178), iron concentration (146, 182), pH (189), exposure to oxidative stress (282), and phosphate starvation (211). Similarly, the biology of rhizosphere-colonizing bacteria has been studied using simplified in vitro approaches. For example, root exudates have been collected from plant roots and used to study the bacterial response to root-derived factors (68, 176); the response of bacteria to other inhabitants of the rhizosphere has also been studied (294). Likewise, in vitro studies have proved useful for identifying host signal molecules triggering the onset of *Agrobacterium tumefaciens* pathogenesis (59, 61) and *Rhizobium* symbiosis (25, 207) upon interaction with plants.

Despite the value of in vitro studies, there is no escape from the fact that the vast majority of microbes exist in complex, dynamic environments that cannot be reproduced in the laboratory. For microbes, irrespective of their life style, there is growing recognition of the need to understand their function in the very environments that they inhabit and thus, ultimately, the causes of their ecological success.

Analysis of ecological success is far from straightforward: it is a complex and multidimensional phenotype determined by interconnected regulatory pathways involving both individual genes and gene networks. Natural selection, which is largely responsible for shaping the determinants of ecological success, does so by operating on interacting systems (more so than on single genes) to generate specific morphologies, physiologies, and behaviors. With this in mind, the value of different experimental approaches can be assessed.

Both bottom-up (genes to population) and top-down (population to genes) approaches have been used. The bottom-up approach is commonly used for studies of bacteria, although it is rarely pursued to the population level. The typical genes-to-phenotype strategy involves identification of traits on the basis of gene inactivation (143). This is a powerful approach that has been fundamental to the majority of advances in molecular microbiology, but, despite its power, insertional mutagenesis is not always appropriate for the analysis of phenotypes as complex as ecological performance. For most organisms, in most environments, there is no primary determinant of ecological performance; this is because it is determined by complex epistatic interactions among many different gene products that each have a long evolutionary history. Traits having the greatest effect on ecological performance are likely to be those that show subtle quantitative variation, and such traits are unlikely to produce "defective" phenotypes when inactivated (143).

Recent advances in gene fusion technologies provide an alternative way to study complex phenotypes. Rather than identifying genes on the basis of function loss, ecologically significant genes can be identified on the basis of their positive contribution to a specific phenotype. A study that aims to understand the mechanistic basis of ecological performance in bacteria colonizing a specific host might, therefore, begin by identifying those genes that are induced in the host environment. One advantage of this approach is that it considers bacteria as integrated organisms rather than as a toolbox of independent genes and phenotypes.

Bacterial gene expression can be determined by direct or indirect measurements of mRNA levels. Reporter gene fusions provide simple indirect methods for assaying transcription by placing a gene that encodes a product that can be readily assayed under the control of the promoter of interest. Two such reporters are *lacZ* (which encodes β-galactosidase) (116) and *gusA* (which encodes β-glucuronidase) (247). While reporters such as *lacZ* and *gusA* have been used most extensively to study gene expression in vitro, both of these reporters have also been used to study expression in complex environments, such as within the environment of living cells. However, improved reporters that encode luminescent (e.g., *lux*) or fluorescent (e.g., *gfp*) proteins have greatly increased the utility of transcriptional reporters to the extent that expression of single cells in complex environments can be studied (34, 41, 53, 85, 242).

In the past decade, many different techniques have been developed to study bacterial genes that are expressed during growth in specific and complex ecological niches (47, 138, 220). In this article we discuss the promoter-trapping techniques differential fluorescence induction (DFI) (279) and in vivo expression technology (IVET) (156), which have been used to identify and study genes showing elevated levels of expression in complex environments. In addition to the information these genes provide about the way that an organism perceives its environment, genes activated in a specific niche are likely to encode (or contribute toward) traits that are important determinants of ecological performance in that environment (201, 212). Complementary strategies such as signature-tagged mutagenesis (STM) (99), differential display using arbitrarily

primed PCR (69, 172), subtractive and differential hybridization (111, 112), and selective capture of transcribed sequences (SCOTS) (86), are reviewed elsewhere (37, 94, 96, 154).

IN VIVO EXPRESSION TECHNOLOGY

Development of In Vivo Expression Technology

More than 15 years ago, Osbourn et al. designed the experimental approach now widely known as in vivo expression technology (190). To isolate *Xanthomonas campestris* genes induced during infection of turnips, the authors used a promoter trap containing a promoterless chloramphenicol resistance gene. In 1993 Mahan et al. (156) described a modified promoter trap and coined the term in vivo expression technology (IVET). This allowed the identification and subsequent analysis of *Salmonella* genes expressed during infection of mice.

IVET (Fig. 1) is a promoter-trapping technique that selects microbial promoters active in a specified niche, for instance, during the interaction of a microorganism with its host. The first component of IVET is a conditionally compromised strain of the microorganism of interest that is mutated in a gene encoding an essential growth factor (*egf*) (220). The mutant strain is not able to sustain growth in the environment under study unless the *egf* gene is expressed. The second component of IVET is a plasmid carrying the promoter trap composed of the promoterless *egf* gene and a linked reporter gene (*rep*). Bacterial DNA is cloned randomly into the promoter trap and integrated in the chromosome of the *egf* mutant strain. Promoters that are specifically induced in the wild are identified by the ability to drive expression of the promoterless *egf* gene in this environment. This results in complementation of the mutation and, hence, in growth under the conditions encountered in the specified niche. To eliminate fusions with a "constitutive" promoter, recovered bacteria are screened for expression of the linked reporter gene (*rep*) on a general growth medium.

Mahan and colleagues (156) devised the IVET concept to meet three important criteria. First of all, integration of a single copy of the transcriptional fusions into the chromosome avoids gene dosage effects inherent in multicopy plasmid vehicles. However, several authors applied IVET with a promoter trap provided on a stably maintained plasmid. Second, the integration of fusions by a single recombination event in the host chromosome generates a duplication of the cloned DNA, thereby retaining a functional copy of the wild-type gene and avoiding the loss of virulence factors or disruption of genes that may be essential for survival in the wild. Third, the reporter gene for screening promoter activity in vitro, in most cases *lacZ*, *gusA*, or *gfp*, enables the monitoring of promoter activity in vitro and in the wild using a chromogenic substrate or fluorescence detection.

Bacteria harboring promoters that are specifically active in the wild are isolated from the specified niche, and the transcriptional fusions are rescued from the genome by standard molecular cloning procedures. However, this is laborious and can also be problematic. Therefore, alternative methods to recover fusions from the genome have been devised. One method is to recover the fusion by transduction using a suitable phage, e.g., bacteriophage P22 in the case of *Salmonella* spp. (155), but transducing phages are not widely available. A more generally applicable procedure to rescue chromosomally integrated plasmids is conjugative cloning (219). A helper plasmid supplies the genetic loci necessary for mobilization of the integrated plasmid into a suitable *Escherichia coli* host (220).

The IVET screening by Mahan et al. (156) relied on a *purA* or *thyA* null mutation, resulting in purine and pyrimidine auxotrophy, respectively, that greatly attenuated the growth of *Salmonella enterica* serovar Typhimurium during mouse infection (98, 156). Since then, a wide variety of genes encoding essential growth factors (Table 1), as well as different reporter genes have been used.

Selection Strategies in IVET

Several variations on the original IVET theme have emerged. These IVET variants involve selection strategies based upon auxotrophy, antibiotic resistance, or recombination events resulting in the excision of a genetic marker. In addition, some highly specific IVET selection strategies have also been devised.

Auxotrophy-based selection. The auxotrophy-based selection strategy has been widely used in IVET screenings. All IVET studies based on this type of selection require a mutant strain defective in growth in the wild. This growth defect can be complemented by expression of the promoterless essential gene provided on the promoter trap.

As mentioned above, the first studies under the IVET moniker used auxotrophic *Salmonella enterica* serovar Typhimurium mutants defective in the de novo biosynthesis of purine or pyrimidine nucleotides combined with promoter traps supplying a promoterless *purA* or *thyA* gene, respectively, to complement growth of the *Salmonella enterica* serovar Typhimurium mutants in the wild (98, 156). The *purA*-based selection strategy was also used to identify *Pseudomonas aeruginosa* genes specifically induced during infection of mice (93). In another IVET study, purine auxotrophy of *P. aeruginosa* was obtained using a *purEK* mutant strain (289, 290).

Since not all bacteria show defective growth upon *purA* mutation and it is difficult to obtain a *purA* mutant strain for some microorganisms, several research groups used other essential genes. In principle, any biosynthetic gene that is necessary for growth in the wild can be used. The only prerequisite is that the auxotrophy cannot be complemented by metabolites retrieved from the occupied niche. Nevertheless, the gene to be mutated has to be chosen after careful consideration. For instance, when bacteria are able to reside intracellularly in host tissue, it has to be taken into account that nonsecreted host metabolites might also complement the auxotrophy.

Several authors adapted the auxotrophy-based selection strategy and used other essential metabolic genes (Table 1): *panB*, involved in pantothenate biosynthesis (218); *dapB* or *asd*, involved in diaminopimelate biosynthesis (63, 78, 95, 224, 245); *metXW* (159) or *trpEG* (26), necessary for methionine and tryptophan biosynthesis, respectively; *inhA*, required for mycolic acid biosynthesis (56); *pyrB* or *thyA*, necessary for de novo biosynthesis of pyrimidine nucleotides (140, 156); *galU*, involved in galactose metabolism (133); or *ribBAH*, involved in riboflavin biosynthesis (76). IVET was also applied to study infection of mice by the pathogenic fungus *Histoplasma capsu-*

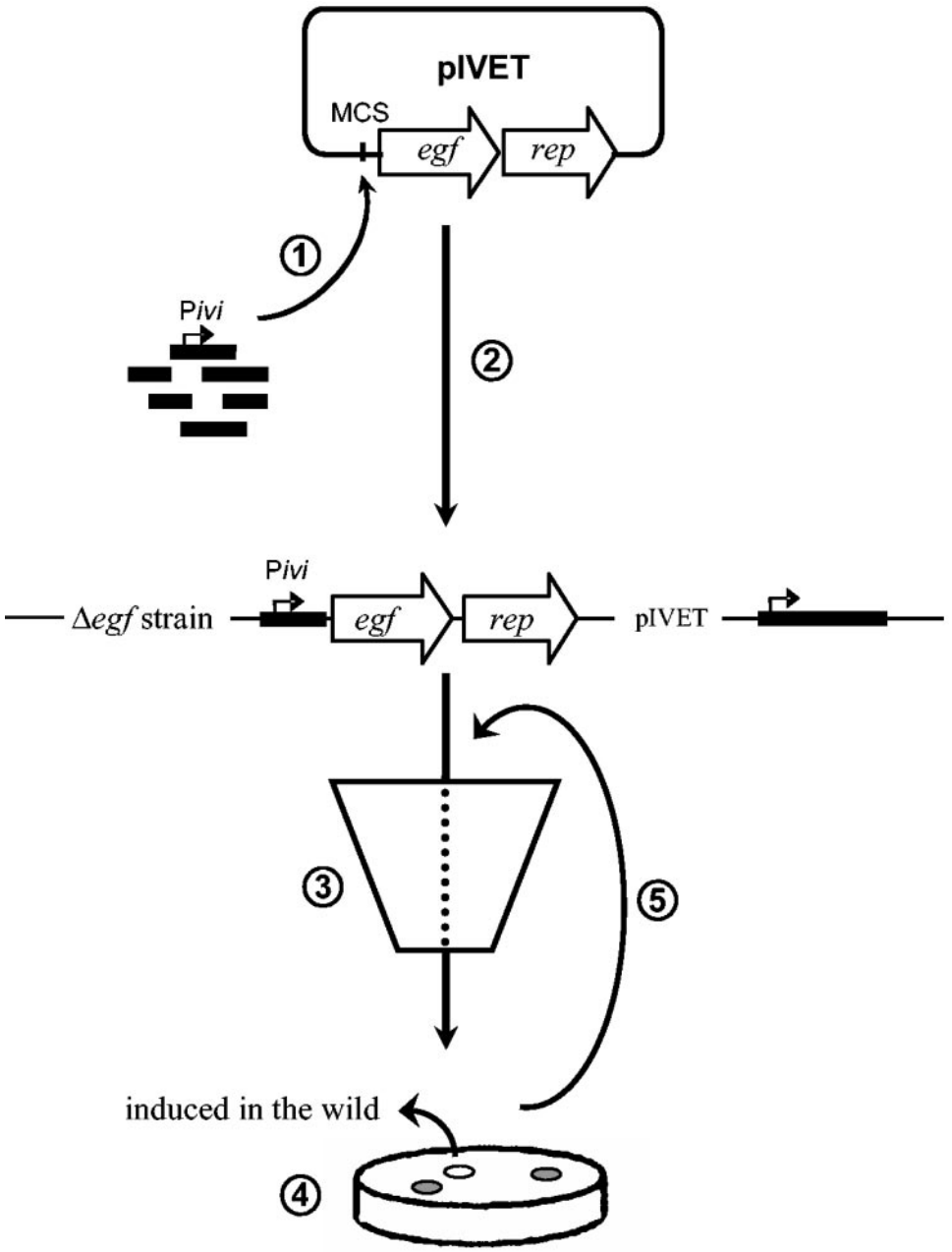


FIG. 1. Schematic representation of the basic IVET strategy. This strategy involves the construction of a conditionally compromised strain that is mutated in a gene encoding an essential growth factor (*egf*). This mutant strain is not able to grow in the environment under study. The second component of IVET is the promoter trap, consisting of a promoterless *egf* gene and a transcriptionally linked reporter gene (*rep*). Bacterial DNA is cloned randomly into the promoter trap (step 1) and integrated in the chromosome of the *egf* mutant strain (step 2). Only in strains that carry a promoter active in the specified niche can the *egf* mutation be complemented (step 3). After selection in this environment, bacteria are reisolated and spread on a general growth medium that is suitable for monitoring reporter gene activity in vitro (step 4). Accordingly, constitutive promoters are distinguished from promoters that are specifically induced in the wild. Colonies bearing the latter type of transcriptional fusion are subjected to a second IVET screening to eliminate false positives (step 5).

latum. In this case, uracil auxotrophy was created by mutating the *ura5* gene (226).

Antibiotic resistance-based selection. As it is not always easy or possible to construct an auxotrophic mutant, IVET selection based on antibiotic resistance, using antibiotic resistance genes as reporter genes (Fig. 2), is an important variant that expands the applications to a wider variety of microorganisms. While extending the utility of IVET, the use of antibiotic selection typically requires dosing the environment of interest with antibiotic, which inevitably changes aspects of the biological niche studied with implications for the spectrum of genes recovered.

Osbourn et al. (190) used an "IVET avant la lettre," based on antibiotic selection to isolate genes of the plant pathogen *Xanthomonas campestris* induced during infection of turnip.

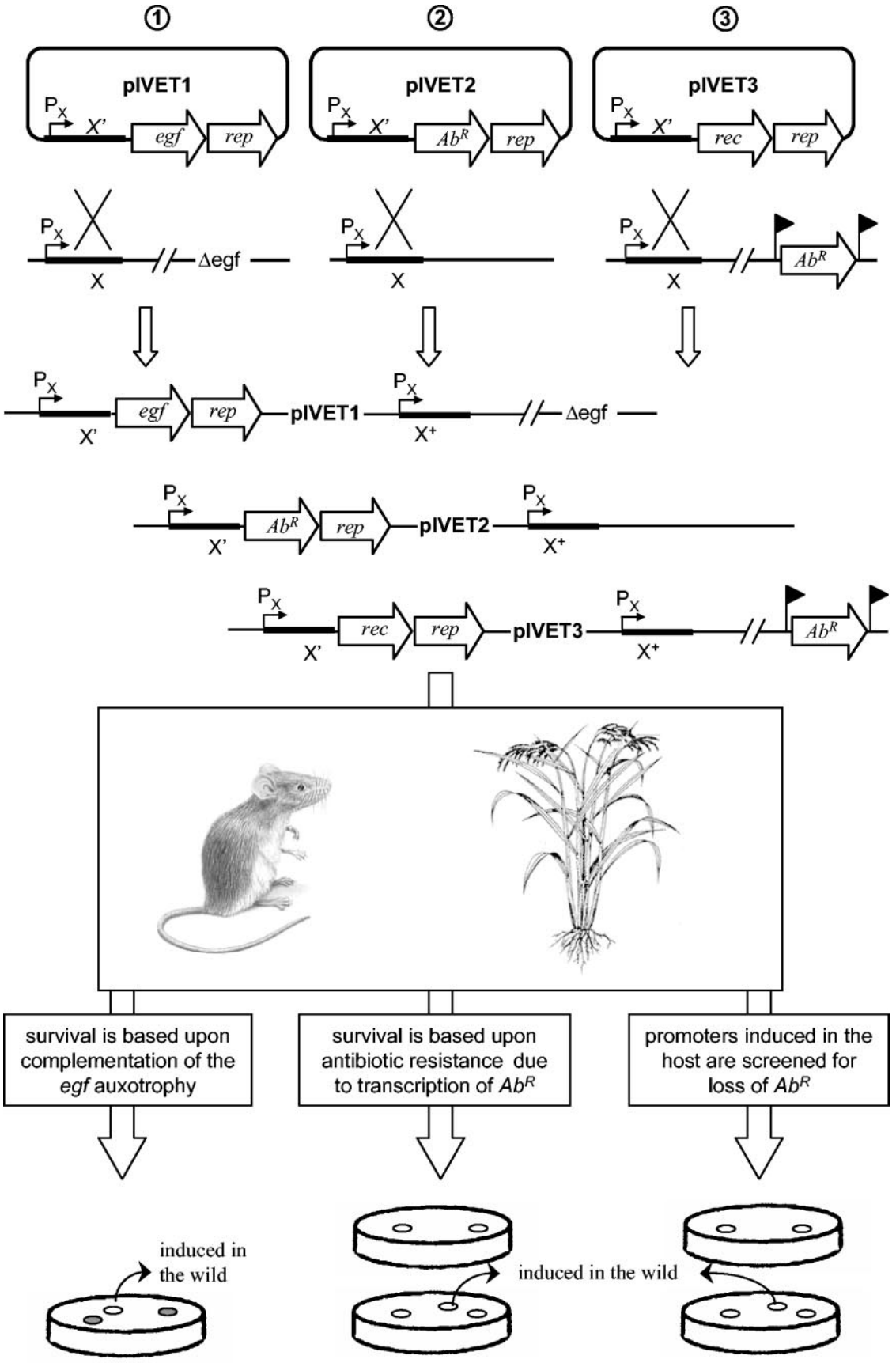
TABLE 1. Overview of applications of IVET to isolate microbial genes upregulated in complex niches

Category and gene	Function	Microorganism	Host or environment	Reference(s)
Auxotrophy-based selection				
purA	De novo purine nucleotide biosynthesis	*Salmonella enterica*	Mouse	98, 156, 264
		Pseudomonas aeruginosa	Mouse	93
purEK	De novo purine nucleotide biosynthesis	*Pseudomonas aeruginosa*	Burned tissue	92
			Biofilm	67
			Mouse	290
thyA	De novo thymidine nucleotide biosynthesis	*Salmonella enterica*	Mouse	156
pyrB	De novo pyrimidine nucleotide biosynthesis	*Pseudomonas putida*	*Phytophthora parasitica*	140
ura5	Uracil biosynthesis	*Histoplasma capsulatum*	Mouse	226
asd	Diaminopimelate biosynthesis	*Pseudomonas aeruginosa*	Mouse	95
		Pseudomonas putida	Maize	63
		Shigella flexneri	Macrophage	244
dapB	Diaminopimelate biosynthesis	*Pseudomonas fluorescens*	Sugar beet	78, 302, 303
		Pseudomonas fluorescens	Bulk soil	245
		Pseudomonas stutzeri	Rice	224
panB	Pantothenate biosynthesis	*Pseudomonas fluorescens*	Sugar beet	218
trpEG	Tryptophan biosynthesis	*Ralstonia solanacearum*	Tomato	26
galU	Galactose metabolism	*Klebsiella pneumoniae*	Mouse	133
inhA	Mycolic acid biosynthesis	*Mycobacterium tuberculosis*	Macrophage	56
ribBAH	Riboflavin biosynthesis	*Actinobacillus pleuropneumoniae*	Pig	76
Antibiotic resistance selection				
cat	Chloramphenicol resistance	*Xanthomonas campestris*	Turnip	190
		Burkholderia pseudomallei	Macrophage	239
		Shigella flexneri	Macrophage	14
		Escherichia coli	Mouse	126, 191
		Helicobacter pylori	Mouse	8
		Salmonella enterica	Mouse	98, 157
		Yersinia enterocolitica	Mouse	83, 300
		Streptococcus gordonii	Rabbit	127, 191
		Yersinia ruckeri	Fish	65
erm	Erythromycin resistance	*Lactobacillus reuteri*	Mouse	285
		Streptococcus suis	Pig	254
tet	Tetracycline resistance	*Porphyromonas gingivalis*	Mouse	141, 298
kan	Kanamycin resistance	*Pasteurella multocida*	Mouse	108
RIVET				
tnpR	Site-specific recombinase	*Vibrio cholerae*	Rabbit/mouse	32, 137, 174, 191
		Staphylococcus aureus	Mouse	152
cre	Site-specific recombinase	*Salmonella enterica*	Epithelial cells	5
		Lactobacillus plantarum	Mouse	24
FLP	Site-specific recombinase	*Candida albicans*	Mouse	262
System-specific selection				
bacA	Membrane protein necessary for bacteroid differentiation	*Sinorhizobium meliloti*	Alfalfa	188
hly	Listeriolysin	*Listeria monocytogenes*	Mouse	55, 77
metXW	Methionine biosynthesis	*Pseudomonas syringae* pv. *syringae*	Bean	159
hrcC	Component of TTSS	*Pseudomonas syringae* pv. *tomato*	*Arabidopsis thaliana*	16
gfp	Green fluorescent protein	*Erwinia chrysanthemi*	Spinach	299

The promoter trap was provided on a stably maintained plasmid and consisted of a promoterless *cat* gene, encoding chloramphenicol acetyltransferase. The chloramphenicol resistance gene was used as a reporter for both in vitro and host-induced promoter activity by screening the bacteria for chloramphenicol sensitivity and resistance, respectively.

Later, the *cat* gene was also used in IVET studies of *Shigella flexneri* (14), *Salmonella enterica* serovar Typhimurium (98, 157), *Helicobacter pylori* (8), *Yersinia enterocolitica* (300), *Yersinia ruckeri* (65), *Streptococcus gordonii* (127), *Escherichia coli* (126), and *Burkholderia pseudomallei* (239). Bacteria harboring promoters that are specifically induced in the wild were selected by administrating chloramphenicol to the host.

The use of antibiotic resistance genes as reporter genes in


①
②
③
pIVET1
pIVET2
pIVET3
P
X'
egf
rep
Ab^R
rec
Δegf
X
X+
survival is based upon complementation of the egf auxotrophy
survival is based upon antibiotic resistance due to transcription of Ab^R
promoters induced in the host are screened for loss of Ab^R
induced in the wild
induced in the wild

IVET studies is not limited to *cat*. To study *Porphyromonas gingivalis* virulence in mice, the promoterless *tet* gene was used, conferring tetracycline resistance (141, 298). Induced gene expression during pig infection by *Streptococcus suis* (254, 255) and mouse infection by *Lactobacillus reuteri* (285) was analyzed by an erythromycin resistance-based screening. And gene expression by *Pasteurella multocida* infecting mice was explored using a kanamycin resistance reporter gene (108).

Recombinase-based selection. Both auxotrophy-based and antibiotic resistance-based selection have to cope with the inability to isolate transiently or weakly expressed genes. These disadvantages are circumvented by a second major modification of IVET screening, recombinase-based in vivo expression technology (RIVET). RIVET is based on the activation of a site-specific DNA resolvase and was initially used to identify *Vibrio cholerae* genes induced during infection of mice (32, 137). The resolvase used, TnpR from Tnγδ, is able to mediate recombination between two specific target sequences, the so-called *res1* sites, and consequently slice out the interjacent DNA fragment from the genome.

In the first RIVET application, a tetracycline resistance gene (*tet*) was chosen as the reporter gene and was integrated into the chromosome, flanked by two *res1* sites. The promoterless *tnpR* gene was provided on the promoter trap. Active promoters direct transcription of *tnpR*, and the activity of the resolvase results in excision of the reporter gene from the genome. By selection for tetracycline resistance during construction of the library, promoters that are active in vitro are discarded. After reisolation from the host, bacteria are screened for tetracycline sensitivity, and promoters active during interaction with the host are retained. The RIVET strategy has also been validated to study *Staphylococcus aureus* infection of mice (152). In this case, a kanamycin resistance gene was used as reporter gene and was integrated into the chromosome, flanked by two *res1* sites.

A similar system was used in a RIVET strategy to study *Salmonella enterica* serovar Typhimurium infection of mice (5). This system consists of a promoterless derivative of *cre*, encoding the phage P1 recombinase, carried on the promoter trap. The targets of the Cre recombinase are two chromosomally integrated *loxP* sites flanking the *npt* gene, conferring kanamycin resistance.

RIVET is applicable to many microorganisms, even those that are difficult to manipulate since only the reporter gene flanked by recognition sites has to be integrated into the chromosome. A Cre-based RIVET system was devised by Bron et al. (24) for lactic acid bacteria. To study infection of mice by the fungal pathogen *Candida albicans*, Staib et al. devised a RIVET system consisting of an Flp recombinase and a genetic marker, conferring resistance to mycophenolic acid, flanked by the specific recognition sites for the recombinase (262).

System-specific selection. It can be of interest to identify genes (promoters) differentially expressed during a particular stage of the interaction between a bacterium and its eukaryotic host. To this end, dedicated IVET strategies can be devised in which the promoter trapping gene encodes an "essential interaction factor" (*eif*) required at a specific stage of the interaction. Bacteria are screened for the ability to establish a firm interaction with the host. It is therefore necessary that the establishment of the microbe-host interaction result in a scorable host phenotype, such as cell lysis, plant disease, or symbiosis.

For example, a specific IVET selection strategy was devised to isolate *Sinorhizobium meliloti* genes that are specifically induced in the early stages of symbiosis (188). In this study, the *bacA* and *gusA* genes were used as reporter genes to assess host-induced and in vitro promoter activity, respectively. BacA is an integral membrane protein that affects the degree of modification of the lipopolysaccharides. BacA is required for intracellular infections during *Sinorhizobium meliloti* plant symbiosis and *Brucella abortus* animal pathogenesis (64). BacA is also necessary for differentiation of *Sinorhizobium meliloti* into nitrogen-fixing differentiated cells (bacteroids) (109). Only when active promoters are inserted in the promoter trap will the *bacA* gene be expressed, resulting in the differentiation process. Nitrogen-fixing nodules containing bacteroids can readily be distinguished from non-nitrogen-fixing nodules by macroscopic observation. In this way, the screen targets genes that are expressed after the initiation of nodulation but before bacteroid differentiation and nitrogen fixation take place. Isolation of genes known to be involved in nodulation (e.g., *nifS*) suggests that the strategy functions as expected. Moreover, it enabled identification of genes that were not previously associated with nodulation (188).

A similar strategy was developed to isolate genes involved in the early stage of *Pseudomonas syringae* pv. *tomato* infection of *Arabidopsis thaliana* leaves (16). This IVET consists of a *Pseudomonas syringae hrcC* mutant strain with a defi-

FIG. 2. Schematic overview of the three main IVET selection strategies. Depending on the chosen IVET selection, the promoter trap contains a promoterless reporter gene (*rep*) transcriptionally linked to (1) a promoterless *egf* gene, encoding an essential growth factor (auxotrophy-based selection); (2) a promoterless Ab^R gene, conferring antibiotic resistance (antibiotic resistance-based selection); or (3) a promoterless site-specific recombinase gene (*rec*), which, when expressed, will splice out the antibiotic resistance (Ab^r) gene that is integrated elsewhere in the bacterial genome. Fusion libraries are constructed by ligating random genomic fragments (designated gene *X*) into the IVET vector of choice. Subsequently, the fusion library is transferred to an auxotrophic (*egf*) mutant strain (1) or a strain harboring the Ab^r gene flanked by recognition sites for the recombinase (indicated by flags) (3) in the case of auxotrophy-based and RIVET selection, respectively. After transfer of the transcriptional fusions into the microorganism of interest, the suicide plasmid is, in most cases, integrated into the chromosome at the sites of homology to gene *X*, thereby creating a merodiploid and retaining a functional copy of gene *X* (indicated with X^+). In the case of RIVET, prescreening is required to remove strains harboring in vitro active gene fusions by selecting for Ab^R during construction of the fusion library. Subsequently, strains carrying the fusions are passed through the specific environment of interest and collected after a period of time. For antibiotic resistance-based selection, the antibiotic must be administered to the environment at a sufficient dose. Strains containing genes induced in the wild are selected by the ability to sustain growth in the environment (auxotrophy- or antibiotic resistance-based selection) or by screening for the loss of antibiotic resistance after recovery (RIVET selection). In the case of auxotrophy-based and antibiotic resistance-based selection, constitutive promoters can be discarded by monitoring the activity of the reporter gene in vitro and for antibiotic sensitivity, respectively, on a general growth medium.

cient type III secretion system (TTSS). TTSS is necessary for infection and growth in susceptible plants. Subsequently, a promoterless *hrcC* gene was used as the reporter in the promoter trap. Only genes expressed during establishment of infection can be isolated with this modified IVET. The approach used here proved useful, since 40% of the transcriptional fusions revealed genes already known to be involved in pathogenesis. Validation was obtained by isolation of *hrp*/*hrc* and *avr* genes, encoding proteins of the TTSS, as they are known to be induced upon inoculation and hence during the early stage of infection. A similar system is being developed with a *Xanthomonas campestris* pv. *vesicatoria hrpB1* mutant also defective in expression of a functional TTSS (U. Bonas, personal communication).

To isolate *Listeria monocytogenes* virulence genes, a modified IVET was devised based on *hly*, encoding a hemolysin (listeriolysin) (55, 77). Listeriolysin is a virulence factor absolutely required for intracellular survival and growth in mice. Disruption of *hly* results in the loss of the hemolytic phenotype on blood agar plates and a severe decrease in virulence. Consequently, infection of mice by *hly* mutants can only occur when the *hly* gene provided on the promoter trap (lacking its cognate promoter) is expressed. After isolation of the infecting bacteria, the same reporter gene (*hly*) was used to screen promoter activity in vitro, since hemolysis is apparent as a zone surrounding Hly^+ bacteria on blood agar plates. Again, this modified IVET focuses on genes that are induced and necessary in the early stages of infection rather than genes that enable bacteria to adapt to and survive in the new environment.

In an IVET application to study the plant pathogen *Pseudomonas syringae* pv. *syringae*, a methionine auxotrophy-based selection strategy was devised. In moist plant leaves, the *metXW* mutant used displays normal growth, but shows severely attenuated growth on plants in dry conditions (159). In this way, the auxotrophy-based selection only occurs when plants are transferred to dry growth conditions, and the timing and degree of selection pressure can be altered accordingly. For instance, by growing the plants in wet conditions in the early stages of infection, the conditionally compromised *metXW* mutants are able to grow and establish large populations. The IVET selection regimen is subsequently started by transferring the plants to dry conditions. Therefore, the name habitat-inducible rescue of survival was introduced (159).

The green fluorescent protein (GFP)-based IVET leaf array for identification of plant-upregulated genes in *Erwinia chrysanthemi*, described by Yang et al. (299), does not involve positive in planta selection using an essential growth factor gene. Selection of induced promoters is based upon differences in fluorescence intensity during plant infection and during growth on a general growth medium.

Benefits and Shortcomings of IVET Strategies

A major advantage of IVET is that the genes of interest are isolated from the fusion library by a powerful positive selection strategy (6). This is not possible with STM, for instance. Moreover, with STM there is no detection of virulence factors that are essential for survival in vitro because knocking out these genes results in defective growth (10).

Since the early IVET studies of animal infection by *Salmonella enterica* serovar Typhimurium and *Pseudomonas aeruginosa*, IVET has been adapted and applied to study a wide variety of microorganisms. It is clear from Table 1 that the various IVET selection strategies extended its use to study differential gene expression not only in gram-positive bacteria but also in eukaryotic microorganisms such as *Candida albicans* (262) and *Histoplasma capsulatum* (226).

IVET is not technically demanding and can be applied using standard molecular biology techniques. This means that in contrast to DFI or microarrays, no expensive equipment is required. Another major advantage of IVET is that no extensive knowledge of the genome of the microorganism under study is required to apply the technique. For example, *Yersinia ruckeri* and *Pseudomonas stutzeri* A15 are bacteria for which only a few DNA sequences were analyzed in the past, but IVET proved a useful technique to analyze gene expression in their host environments (65, 224). However, the availability of a (draft) genome sequence of the target microorganisms or close relatives certainly speeds up subsequent characterization of the trapped promoters.

Variations on the original IVET theme (antibiotic resistance-based IVET, RIVET, and DFI) have enabled the study of microorganisms for which straightforward genetic analysis, such as construction of defined mutants, is not readily available. For instance, in eukaryotes the presence of two alleles for each gene hampers mutational analysis, but IVET techniques enabled gene expression analysis of pathogenic fungi in their natural habitat (226, 262).

IVET can be applied to microorganisms residing in ecological niches that are very different in nature. IVET has been successfully applied to study microorganisms residing in animal hosts (ranging from fish, pigs, and chinchillas to mice), in macrophages, in plants, in the rhizosphere, and even in bacteria colonizing an oomycete (Table 1). When the microorganism under study is able to colonize two different host organisms, host-triggered gene expression can be assessed using the same promoter trap library. Comparing the two (different) subsets of host-induced genes provides information about the differences and similarities in the microenvironment of the two host organisms.

IVET is not limited to studying interactions with animal, fungal, or plant hosts, but can be extended for use in other complex environments. IVET was, for instance, used to study *P. aeruginosa* in biofilms with the so-called in-biofilm expression technology (67), or to study differential gene expression of *P. aeruginosa* during infection of burned mice tissues (92). A *dapB*-based IVET system was used to explore the genetic needs for survival of *Pseudomonas fluorescens* Pf0-1 in bulk soil (245). An IVET technique is also being developed to study gene expression of the oil-degrading marine bacterium *Alcanivorax borkumensis* in response to key environmental signals in order to study the bacterial determinants involved in biodegradation of hydrocarbons (82). In addition, an IVET-like strategy has been used to study differential gene expression in different genetic backgrounds. With the so-called identification of transcriptional regulator-activated promoters, the dependence of the transcription of *Mycobacterium tuberculosis* genes on various transcriptional regulators such as sigma factor σ^E could be

analyzed by monitoring the reporter gene activity in a σ^E-overexpressing and a σ^E knockout strain of *Mycobacterium smegmatis* (173).

IVET has many attractive features, but some possible drawbacks have to be considered in the interpretation of the resulting data. First, IVET is not designed to isolate repressed promoters. Second, the subset of genes that are identified depends on the strength of the selection regimen in the wild. In each experimental system, the strength and the method of selection in the wild have to be chosen with consideration. If the selection is too strong, weakly or transiently expressed promoters will not be identified and highly expressed genes will be favored in the screening. On the other hand, a too weak selection in the wild will lead to false positive results. Third, proteins that are expressed constitutively but only activated in the wild (for example, by phosphorylation) are not detected. Fourth, the sets of genes defined as specifically induced in vivo are partially dependent on the "in vitro" growth conditions used to assess whether the reporter gene fusion is inactive outside the environment under investigation. For instance, the composition of the growth medium can significantly impact the expression of genes involved in nutrient acquisition and metabolism. Finally, mutants affected in genes that are isolated with IVET have to be constructed and phenotypically characterized. Only for a limited number of model microorganisms are ordered mutant libraries available that cover the entire sequenced genome.

As IVET can, in principle, be applied to study virtually all culturable microorganisms in their complex environments, it is clear that many researchers benefit from using the IVET strategy to study their favorite bug. The choice of selection strategy is facilitated by the development of the different IVET modifications. However, each specific selection strategy comes with its own advantages and disadvantages.

The major disadvantage of autotrophy-based selection is the need to construct an auxotrophic mutant, and for some microorganisms the tools to achieve this are not (yet) available. However, the nature of the auxotrophic mutation can determine in part the strength of the selection in the wild. When the generated auxotrophy is lethal for actively growing cells but does not impair cell survival, auxotrophy-based selection becomes a very powerful tool since the strength of selection in the wild can be easily adjusted by altering the time lapse between infection and reisolation (220). Whether low or transiently expressed genes will be detected depends on the strength of the selection regimen.

Switching to antibiotic selection avoids the construction of a mutant strain, thereby increasing the applicability of IVET. However, drug administration to the host might interfere with the complex process of interaction, e.g., with the immune defense of the host. Due to the presence of antibiotics, the composition of the natural ecological niche of which the microorganism is part might be altered. Furthermore, antibiotic administration to the host is not always possible, as the host organism itself might be affected by antibiotic treatments, as is often the case with plants (190). Moreover, to study microorganisms that reside within plant tissues, this selection strategy is scarcely suitable since several antibiotics are not translocated to all plant tissues. Once a suitable antibiotic for selection is found, it is important to evaluate the proper dose of antibiotic administration to allow selection of promoters that drive expression of the antibiotic resistance gene. The selection regimen in the wild can be modified easily. Variation of the antibiotic concentration allows isolation of genes that are expressed at different levels. Changing the time of drug administration permits isolation of genes that are expressed at different time points.

The main disadvantage of RIVET is the loss of a positive selection strategy after reisolation from the environment. This screening is rather laborious since isolated microorganisms are tested for antibiotic sensitivity by replica plating. However, Merrell and Camilli (175) solved this problem by inserting, together with an antibiotic resistance gene, a second reporter gene, *sacB*, into the excisable cassette. Its gene product, levansucrase, catalyzes conversion of sucrose into levan, which is toxic for most gram-negative and some gram-positive bacteria and results in defective growth in media containing sucrose. The *sacB* reporter gene can be used for a positive selection because bacteria that contain promoters induced in the wild have lost the reporter gene cassette, thereby enabling growth on media containing sucrose. Another strategy to avoid negative selection with RIVET is the use of a *cat* resistance gene which is disrupted by a *tet* resistance gene flanked by *res* sites (147). With this so-called selectable in vivo expression technology, the nonresolved strains remain resistant to tetracycline, while the resolved strains become resistant to chloramphenicol.

The major advantage of RIVET compared to antibiotic- and auxotrophy-based selection consists in the isolation of weakly or transiently expressed genes. However, the sensitivity of RIVET could also turn into a disadvantage, as genes important in the wild but expressed in vitro at a moderate to high basal level will not be isolated. The gene of study must be transcriptionally silent during strain construction and propagation in vitro. Otherwise, the antibiotic cassette is spliced out during the library construction, and consequently, the bacteria cannot survive the antibiotic selection. For this reason, fine tuning of the RIVET selection strategy was achieved by modulating the ribosome binding site of the promoterless recombinase gene (139). As a result of mutations in this ribosome binding site, translation efficiency at any transcriptional level is decreased, resulting in less sensitive selection.

Promoter traps consisting of *tnpR* alleles with different translation efficiencies render different pools of isolated genes that are induced to some level in the wild due to the lowered sensitivity of RIVET (139). Recently, a tunable RIVET system to study *Vibrio cholerae* infection was also achieved using resolvable cassettes with different efficiencies of excision (191). However, for each *tnpR* allele or resolvable cassette, a separate fusion library has to be constructed, which multiplies the manipulations.

RIVET permits the analysis of spatial and temporal gene expression (139). The expression patterns of the genes of interest can be investigated at different time points of the interaction, at different anatomic sites of the host organism, and even in different hosts or different genetic backgrounds simply by determining the proportion of resistant bacteria versus those sensitive to the antibiotic.

DIFFERENTIAL FLUORESCENCE INDUCTION

Development and Applications

DFI is a promoter-trapping technique that utilizes the green fluorescent protein (GFP) as a selectable marker to monitor promoter activity. In combination with fluorescence-activated cell sorting (FACS), DFI allows high-throughput screening of gene expression in microorganisms in a semiautomated way. Subsequent cycles of FACS screening result in the enrichment of clones containing genes specifically induced in the conditions under study (278).

DFI was originally designed to isolate acid-inducible genes in *Salmonella enterica* serovar Typhimurium (278, 280). This technique was subsequently adapted to study induction of *Staphylococcus aureus* and *Streptococcus pneumoniae* gene expression by in vitro stimuli that mimic the host environment, such as temperature shift, increased osmolarity, iron limitation, increased acidity, presence of competence stimulatory peptide, and cation starvation (13, 160, 237).

DFI studies of *Salmonella enterica* serovar Typhimurium, *Mycobacterium marinum*, and *Listeria monocytogenes* showed that DFI is not limited to studies of the effect of in vitro stimuli mimicking the host, but also enables analysis of gene expression during infection of macrophages (11, 221, 279, 295). Although these studies demonstrated successful sorting of macrophages based on the fluorescence of infecting bacteria, it is worth noting that the bacterial population within an infected macrophage is heterogeneous, which might lead to false positive results. However, it was possible to apply DFI to study *Streptococcus pneumoniae* infection when the pathogen was isolated from host body fluids, such as blood (160). Using two-color flow cytometry, Bumann (30) successfully analyzed gene expression of *Salmonella* organisms isolated from mouse Peyer's patches. Recently, the use of DFI was extended to explore differential gene expression of plant-associated bacteria such as *Rhizobium leguminosarum* (3), *Pseudomonas syringae* (35), and *Bacillus cereus* (57).

Benefits and Shortcomings of DFI

The benefits of DFI include semiautomated screening of large populations and the ability to change the sensitivity of the selection by simply altering the fluorescence threshold (278). Moreover, DFI is highly reproducible and enables integration of high-throughput screening and genomics (265). The use of GFP enables visualization of gene induction and the analysis of promoter activity on the single-cell level, which can be useful since heterogeneity of gene expression in a population increases with the complexity of the environment (18, 281).

In contrast to IVET, transcriptional fusions are not integrated into the chromosome but are provided on plasmids because single-copy *gfp* expression results in sufficiently intense fluorescence for accurate measurement only when driven by a strong promoter. In addition, the use of plasmids facilitates the isolation of in vivo-induced promoters. However, the use of multicopy plasmids prevents detection of context-dependent or topology-dependent effects of gene regulation.

DFI shares with IVET the caveats that are inherent to promoter trap approaches, such as the inability to detect genes that are regulated posttranscriptionally and the need to construct and analyze mutated target genes to assess their role in the wild (154). However, DFI shows additional disadvantages intrinsic to the technology. Flow cytometric analysis and sorting can be hampered by aggregation of bacteria or macrophages. Additional problems arise with the isolation and fluorescence quantification of bacteria that are isolated from infected host tissues because of the prevalence of background fluorescent particles (138, 278).

Other disadvantages are associated with the use of GFP, such as restrictions to the pH range in the studied environment, oxygen requirement for fluorophore development, and the absence of signal amplification. Due to the nonlinearity of fluorescent signals, it is necessary to calibrate and determine the linear range of the signal for each experiment to allow quantification of gene expression (180). However, most of these problems have been solved by the technological advances made concerning maturation, fluorophore development, fluorescence intensity, and spectral properties of GFP (42, 43, 258, 276).

OVERVIEW OF IVET- AND DFI-ISOLATED GENES

The application of IVET and DFI promoter-trapping techniques has allowed isolation of the promoters of many microbial genes that are specifically induced in complex environments. Identification of such genes is instrumental to unraveling microbial life in its natural habitat. Most IVET studies reported to date are unlikely to reflect a comprehensive view of the genes specifically transcribed in the wild. Nevertheless, the reported studies are a significant step forward in understanding how microorganisms respond to diverse environmental niches and provide clues as to the determinants of ecological success.

In Table 2, host-induced genes identified with IVET or DFI are classified in 9 functional groups. Most of the data in Table 2 were obtained from IVET studies of bacterial pathogens during infection of a mammalian host. In most cases a murine infection model was used to explore host-induced gene expression, but the interaction of animal pathogens with fish, pigs, rabbits, and chinchillas was also studied with promoter traps. In recent years, however, the IVET technique has found wider application for exploration of in planta gene expression of phytopathogenic bacteria as well as in nonpathogenic systems of bacterial interaction with crop plants (alfalfa, sugar beet, rice, and maize). In the majority of the studies, γ-proteobacterial members (17 species) were covered, mainly enterobacteria (seven species) and *Pseudomonas* (five species). Among the gram-positive bacteria, most data originate from members of the firmicutes (six species), whereas the use of IVET to study actinomycetes has only been reported for *Mycobacterium tuberculosis*. The data of 11 DFI studies of microbe-host interactions are incorporated in Table 2. DFI was used almost exclusively to study animal pathogens, mostly belonging to the γ-Proteobacteria (three species), firmicutes (three species), and mycobacteria (two species).

When interpreting the data from Table 2, one should be aware that (i) although the listed genes are upregulated in the wild, ecological significance has been unequivocally demonstrated by mutant analysis for relatively few, and (ii) most of the listed gene assignments are tentative identifications based

TABLE 2. Promoters of genes that are upregulated in microorganisms during interaction with a eukaryotic host[a]

Class	Function and gene or fusion	Protein function; possible role in host interactions	Organism	Host or environment	Selection	Reference
Class I: genes involved in motility or chemotaxis						
	Flagellum/pilus biosynthesis					
	fliI	Flagellum-specific ATP synthase	*V. cholerae*	Mouse	RIVET	191
	fliF	Flagellar M-ring protein	*P. fluorescens*	Sugar beet	*dapB*	78
	fliF	Flagellar M-ring protein	*S. suis*	Pig	*erm*	254
	α-*flaA*	Antisense transcript—subunit flagellin	*V. cholerae*	Mouse	RIVET	32
	α-*fliM*	Antisense transcript—flagellar C-ring protein (switch complex)	*P. stutzeri*	Rice	*dapB*	a
	Rsc0726	Type IV fimbrial biogenesis (PilW-related protein)	*R. solanacearum*	Tomato	*trpEG*	26
	Chemotaxis					
	iviIV	Chemotaxis receptor protein (MCP)	*V. cholerae*	Mouse	RIVET	32
	vca0773	Chemotaxis receptor protein (MCP)	*V. cholerae*	Mouse	RIVET	191
	vc1535	Chemotaxis receptor protein (MCP)	*V. cholerae*	Mouse	RIVET	191
	trg	Chemotaxis receptor protein (MCP)	*E. chrysanthemi*	Spinach	*gfp-LA*	299
	cheR	Chemotaxis protein (methyltransferase)	*E. chrysanthemi*	Spinach	*gfp-LA*	299
	cheY	Two-component response regulator	*P. aeruginosa*	Mouse	*purEK*	290
	cheY	Two-component response regulator	*R. solanacearum*	Tomato	*trpEG*	26
	α-*cheV*	**Antisense transcript—chemotaxis protein**	***V. cholerae***	**Mouse**	**RIVET**	**32**
	α-*mcp*	Antisense transcript—chemotaxis receptor protein (MCP)	*P. stutzeri*	Rice	*dapB*	224
Class II: genes involved in nutrient scavenging						
	Metal ion acquisition					
	Siderophore synthesis					
	irp2	Yersiniabactin synthetase subunit HMWP2	*Y. enterocolitica*	Mouse	*cat*	300
	iucA	Aerobactin synthesis	*K. pneumoniae*	Mouse	*galU*	133
	entF	Enterobactin synthesis	*S. enterica*	Mouse	*cat*	98
	rucC	Ruckerbactin synthesis	*Y. ruckeri*	Fish	*cat*	65
	pvdD	Pyoverdine synthetase	*P. aeruginosa*	Mouse, rat	*purA*	93
	pvsA	Pyoverdine synthetase	*P. fluorescens*	Sugar beet	*dapB*	78
	Siderophore receptor and uptake					
	fepA	Ferrienterobactin receptor	*K. pneumoniae*	Mouse	*galU*	133
	fptA	Pyochelin receptor	*P. aeruginosa*	Mouse	*purEK*	290
	fyuA	Yersiniabactin receptor	*Y. enterocolitica*	Mouse	*cat*	300
	ufrA	Siderophore receptor	*P. fluorescens*	Sugar beet	*dapB*	78
	foxA	Siderophore receptor	*Y. enterocolitica*	Mouse	*cat*	300
	fhuA	Ferric hydroxamate receptor	*S. enterica*	Mouse	*purA*	98
	fhuA	Ferric hydroxamate receptor	*S. flexneri*	Epithelial cells	DFI	233
	ivi10	**Putative siderophore receptor**	***P. gingivalis***	**Mouse**	***tet***	**298**
	rupA	Ruckerbactin receptor	*Y. ruckeri*	Fish	*cat*	65
	iviVII	TonB complex protein (ExbB-like)	*Y. ruckeri*	Fish	*cat*	65
	rupDGC	Siderophore ABC transporter protein	*Y. ruckeri*	Fish	*cat*	65
	Siderophore-independent iron transport					
	fbpA	Periplasmic iron binding protein of ABC transporter	*B. abortus*	Macrophage	DFI	62
	vc0202	Periplasmic iron binding protein of ABC transporter	*V. cholerae*	Mouse	RIVET	191
	yfeA	Periplasmic (chelated) iron-binding protein of ABC transporter	*E. chrysanthemi*	Spinach	*gfp-LA*	299
	hemT	Periplasmic hemin binding protein of ABC transporter	*B. pseudomallei*	Macrophage	*cat*	b
	hmuT	Periplasmic hemin binding protein of ABC transporter	*E. chrysanthemi*	Spinach	*gfp-LA*	299
	vc0201	ATP binding protein of ABC transporter	*V. cholerae*	Mouse	RIVET	191
	vca0687	ATP binding protein of ABC transporter	*V. cholerae*	Mouse	RIVET	191
	yfuB	Permease of ABC transporter	*Y. enterocolitica*	Mouse	*cat*	300
	vca0203	Permease of ABC transporter	*V. cholerae*	Mouse	RIVET	191

Continued on following page

TABLE 2—*Continued*

Class	Function and gene or fusion	Protein function; possible role in host interactions	Organism	Host or environment	Selection	Reference
	hmuU	Permease of hemin ABC transporter	*E. chrysanthemi*	Spinach	*gfp-LA*	299
	hmuS	Hemin degrading protein	*E. chrysanthemi*	Spinach	*gfp-LA*	299
	sitABCD	**ABC transporter (Fe^{2+}/Mn^{2+} uptake)**	***S. enterica***	**Mouse**	***purA***	**114,156**
	sitA	**Periplasmic binding protein of ABC transporter (Fe^{2+}/Mn^{2+} uptake)**	***S. flexneri***	**Epithelial cells**	**DFI**	**233,234**
	sitC	Permease of ABC transporter (Fe^{2+}/Mn^{2+} uptake)	*Y. enterocolitica*	Mouse	*cat*	83
	Regulation of iron uptake					
	np20	**Ferric uptake regulator (Fur)**	***P. aeruginosa***	**Mouse**	***purEK***	**290**
	Other					
	nex10	**Putative K^+ channel**	***S. meliloti***	**Alfalfa nodules**	***bacA***	**188**
	Rv3237c	K^+ channel	*M. tuberculosis*	Macrophage	*inhA*	56
	trkH	K^+ transport system	*B. abortus*	Macrophage	DFI	62
	kup	K^+ uptake protein	*V. cholerae*	Mouse	RIVET	191
	psaBCA	**Mn^{2+} uptake system**	***S. pneumoniae***	**Mouse**	**DFI**	**160,161**
	mntH	Mn^{2+} transporter	*B. pseudomallei*	Macrophage	*cat*	b
	mgtA	Mg^{2+} transporter	*S. enterica*	Mouse	*cat*	98
	mgtB	Mg^{2+} transporter	*S. enterica*	Mouse	*cat*	98
	ntpJ	Na^+ translocating ATPase	*S. aureus*	Mouse	RIVET	152
	Rsc1951	Solute/Na^+ symporter	*R. solanacearum*	Tomato	*trpEG*	26
	vca2705	Solute/Na^+ symporter	*V. cholerae*	Mouse	RIVET	191
	hoxQ	Ni^{2+} transport/hydrogenase activity	*Y. enterocolitica*	Mouse	*cat*	300
Phosphate acquisition						
	phoA	Alkaline phosphatase	*S. flexneri*	Epithelial cells	DFI	233
	phoB	Response regulator of two-component system	*E. chrysanthemi*	Spinach	*gfp-LA*	299
	phoS	Periplasmic binding protein	*S. enterica*	Macrophages	DFI	279
	phoU	Regulator of transport system	*E. chrysanthemi*	Spinach	*gfp-LA*	299
	pstS	**Periplasmic binding protein of high-affinity ABC transporter**	***S. flexneri***	**Epithelial cells**	**DFI**	**233**
	pstB	ATP-binding protein of high-affinity ABC transporter	*E. chrysanthemi*	Spinach	*gfp-LA*	299
	phnC	ATP-binding protein of ABC transporter	*E. chrysanthemi*	Spinach	*gfp-LA*	299
	phnD	Periplasmic protein of ABC transporter	*E. chrysanthemi*	Spinach	*gfp-LA*	299
	vc0721	Periplasmic phosphate-binding protein	*V. cholerae*	Mouse	RIVET	191
	ppk	Polyphosphate kinase	*E. chrysanthemi*	Spinach	*gfp-LA*	299
Sulfate acquisition						
	cysA	Permease of ABC transporter	*V. cholerae*	Mouse	RIVET	32
	ssuBAC	ABC transporter	*B. abortus*	Macrophage	DFI	62
	cl52	Transporter	*L. monocytogenes*	Macrophage	DFI	295
	cysD	Sulfate adenylate transferase	*M. tuberculosis*	Macrophages	DFI	275
Amino acid acquisition						
	Rsp1575	Periplasmic binding protein of ABC transporter	*R. solanacearum*	Tomato	*trpEG*	26
	ipx46	ATP-binding component of ABC transporter	*P. syringae*	*A. thaliana*	*hrcC*	16
	livMH	Permease for high-affinity transport of branched amino acids	*P. fluorescens*	Sugar beet	*panB*	218
	hutT	Permease for histidine uptake	*P. fluorescens*	Sugar beet	*panB*	218
	iviD	Amino acid transporter	*S. gordonii*	Rabbit	*cat*	127
	sdaC2	Serine transporter	*V. cholerae*	Mouse	RIVET	191
Sugar uptake						
	Phosphotransferase systems					
	celB	Cellobiose PTS, II	*L. monocytogenes*	Mouse	*hly*	77
	pts14C	Cellobiose PTS, IIC	*L. plantarum*	Mouse	RIVET	24
	ptsIBCA	Sucrose PTS, EIIBCA	*L. plantarum*	Mouse	RIVET	24
	pts32BC	Sucrose PTS, EIIBC	*L. plantarum*	Mouse	RIVET	24
	vc0207	Sucrose PTS, EIIBC	*V. cholerae*	Mouse	RIVET	191
	orf5-iviL	Cellobiose PTS	*S. gordonii*	Rabbit	*cat*	127

Continued on following page

TABLE 2—*Continued*

Class	Function and gene or fusion	Protein function; possible role in host interactions	Organism	Host or environment	Selection	Reference
	ptfA	Fructose PTS	*K. pneumoniae*	Mouse	*galU*	133
	ptnA	Mannose PTS	*L. monocytogenes*	Macrophage	DFI	295
	pts37A	Sorbitol PTS, EIIA	*L. plantarum*	Mouse	RIVET	24
	pts19A	*N*-Acetylglucosamine/galactosamine PTS, IIA	*L. plantarum*	Mouse	RIVET	24
	nagE	*N*-Acetylglucosamine PTS, IIABC	*E. chrysanthemi*	Spinach	*gfp-LA*	299
	Other sugar transporters					
	malA	Permease of ABC transporter for maltose uptake	*S. aureus*	Mouse	RIVET	152
	rbsC	Permease of ABC transporter for ribose uptake	*H. influenzae*	Chinchilla	DFI	164
	rbsD	Cytoplasmic ribose-binding protein	*L. plantarum*	Mouse	RIVET	24
	Rsp0536	Transmembrane sugar-proton symporter	*R. solanacearum*	Tomato	*trpEG*	26
	uhpT	Hexose phosphate transporter	*S. flexneri*	Epithelial cells	DFI	233
	rhiT	**Rhamnogalacturonide transporter**	***E. chrysanthemi***	**Spinach**	***gfp-LA***	**299**
	yicJ	Sodium galactoside symporter	*E. chrysanthemi*	Spinach	*gfp-LA*	299
	Uptake of organic acids					
	dctD	Two-component response regulator of C_4-dicarboxylate transport	*P. syringae*	Bean	*metXW*	159
	dctS	Two-component sensor of C_4-dicarboxylate transport	*P. fluorescens*	Sugar beet	*panB*	218
	Rsc1598	Two-component sensor (DctS-homologue)	*R. solanacearum*	Tomato	*trpEG*	26
	lldP	L-lactate permease	*P. fluorescens*	Sugar beet	*dapB*	78
	tctC	Tricarboxylate transport	*Y. ruckeri*	Fish	*cat*	65
	kgtP2	α-ketoglutarate permease	*R. solanacearum*	Tomato	*trpEG*	26
	Uptake of miscellaneous compounds					
	sapA	Peptide ABC transporter	*H. influenzae*	Chinchilla	DFI	164
	cirA	Colicin I receptor	*S. enterica*	Macrophage	*cat*	98
	RT1006	**ATP binding component of ABC transporter**	***S. pneumoniae***	**Mouse**	**DFI**	**160**
	comE	Competence protein (DNA uptake)	*S. suis*	Pig	*erm*	254
	potF2	Putrescine transport protein	*P. fluorescens*	Sugar beet	*dapB*	78
	Transport of unknown substrates					
	Structural components of ABC transporters					
	ivil		*S. gordonii*	Rabbit	*cat*	127
	ivs-6		*S. suis*	Pig	*erm*	254
	orf2		*P. putida*	*Phytophthora parasitica*	*pyrB*	140
	orfU		*S. suis*	Pig	*erm*	254
	Rsc1376		*R. solanacearum*	Tomato	*trpEG*	26
	SPIV013		*S. pneumoniae*	Mouse	DFI	160
	uup		*K. pneumoniae*	Mouse	*galU*	133
	pup-31		*E. chrysanthemi*	Spinach	*gfp-LA*	299
	lp_0299		*L. plantarum*	Mouse	RIVET	24
	rhi-37		*P. fluorescens*	Sugar beet	*dapB*	78
	yoaE		*S. suis*	Pig	*erm*	254
	Periplasmic-binding proteins					
	pup-59		*E. chrysanthemi*	Spinach	*gfp-LA*	299
	p39		*B. abortus*	Macrophage	DFI	62
	Rsc0044		*R. solanacearum*	Tomato	*trpEG*	26
	Components of TRAP-T					
	vc0488	Solute-binding protein	*V. cholerae*	Mouse	RIVET	191
	vc1275	Constituent of TRAP-T carrier	*V. cholerae*	Mouse	RIVET	191
Class III: genes of central intracellular metabolism						
	Intermediary metabolism					
	Glyoxylate pathway					
	aceA	Isocitrate lyase	*M. tuberculosis*	Macrophage	*inhA*	56

Continued on following page

TABLE 2—*Continued*

Class	Function and gene or fusion	Protein function; possible role in host interactions	Organism	Host or environment	Selection	Reference
	aceB	Malate synthase	*Y. enterocolitica*	Mouse	*cat*	300
	aceE	Pyruvate dehydrogenase subunit	*P. syringae*	*A. thaliana*	*hrcC*	16
	pdhC	Pyruvate dehydrogenase subunit	*M. marinum*	Macrophage	DFI	11
	TCA cycle					
	sucA	Subunit of α-ketoglutarate dehydrogenase	*V. cholerae*	Mouse	RIVET	32
	fumC	Fumarase	*R. solanacearum*	Tomato	*trpEG*	26
	fumC	**Fumarase**	***L. monocytogenes***	**Mouse**	***hly***	**77**
	frdB	Fumarate reductase	*Y. enterocolitica*	Mouse	*cat*	83
	ppc	Phosphoenolpyruvate carboxylase	*S. flexneri*	HeLa monolayer	*cat*	14
	ppc	Phosphoenolpyruvate carboxylase	*V. cholerae*	Mouse	RIVET	191
	ppc	Phosphoenolpyruvate carboxylase	*E. chrysanthemi*	Spinach	*gfp-LA*	299
	acnA	Aconitase	*V. cholerae*	Mouse	RIVET	191
	Glycolysis					
	hre-21	Pyruvate kinase	*Y. enterocolitica*	Mouse	*cat*	300
	Gluconeogenesis					
	pckA	Phosphoenolpyruvate carboxykinase	*M. tuberculosis*	Macrophage	*inhA*	56
	pckA	**Phosphoenolpyruvate carboxykinase**	***S. meliloti***	**Alfalfa nodules**	***bacA***	**188**
	Pentose phosphate pathway					
	tal3	Transaldolase	*L. plantarum*	Mouse	RIVET	24
	tkt	Transketolase	*M. tuberculosis*	Macrophages	DFI	275
	tkt	Deoxyxylulose 5′-phosphate synthase/ transketolase	*B. abortus*	Macrophage	DFI	62
	tktI-N	Transketolase	*L. plantarum*	Mouse	RIVET	24
	Other					
	eno	Enolase	*M. tuberculosis*	Macrophage	*inhA*	56
	Lipid/fatty acid synthesis					
	dgk	Diacylglycerol kinase (diacylglycerol recycling)	*P. putida*	*P. parasitica*	*pyrB*	140
	accBC	Biotin carboxyl carrier protein	*S. flexneri*	HeLa monolayer	*cat*	14
	phbA	Acetyl-CoA acetyltransferase	*R. solanacearum*	Tomato	*trpEG*	26
	fadA4	Acetyl-CoA acetyltransferase	*M. tuberculosis*	Macrophage	*inhA*	56
	ephF	Epoxide hydrolase	*M. tuberculosis*	Macrophage	*inhA*	56
	cfa	Cyclopropane fatty acid synthase (membrane modification)	*S. enterica*	Mouse	*cat*	98
	Lipid/fatty acid degradation					
	fadB	Fatty acid oxidation complex (alpha-subunit)	*S. enterica*	Mouse	*cat*	157
	fadB1	Enoyl-CoA hydratase	*B. abortus*	Macrophage	DFI	62
	echA19	Enoyl-CoA hydratase	*M. tuberculosis*	Macrophage	*inhA*	56
	fadB4	3-Hydroxyacyl-CoA dehydrogenase	*M. tuberculosis*	Macrophage	DFI	275
	Rv1144	3-Hydroxyacyl-CoA dehydrogenase	*M. tuberculosis*	Macrophage	*inhA*	56
	fadD	Long-chain fatty acid CoA ligase	*P. syringae*	*A. thaliana*	*hrcC*	16
	fadE	Probable acyl CoA dehydrogenase	*P. fluorescens*	Sugar beet	*dapB*	78
	est2	Acetylesterase	*L. plantarum*	Mouse	RIVET	24
	lipA	Secreted triacylglyceride-specific lipase	*V. cholerae*	Mouse	RIVET	32
	lip	Glycerolester hydrolase	*S. aureus*	Mouse	RIVET	152
	Phospholipid metabolism					
	aas	Acyl acylglycerol phosphoethanolamine acyl transferase	*S. enterica*	Macrophages	DFI	279
	licC	Phosphocholine cytidyltransferase	*H. influenzae*	Chinchilla	DFI	164
	glpQ	Protein D (lipoprotein)	*P. multocida*	Mouse	*kan*	108
	eutR	**Ethanolamine operon regulator**	***E. chrysanthemi***	**Spinach**	***gfp-LA***	**299**
	Sugar metabolism					
	cl 143	UDP-galactose epimerase	*L. monocytogenes*	Macrophage	DFI	295
	rbsK3	Ribokinase	*L. plantarum*	Mouse	RIVET	24
	rbsR	**Transcriptional repressor of ribose operon (LacI family)**	***K. pneumoniae***	**Mouse**	***galU***	**133**
	xylA	Xylose isomerase	*P. fluorescens*	Sugar beet	*panB*	218
	xylA	Xylose isomerase	*L. reuteri*	Mouse	*erm*	285

Continued on following page

TABLE 2—*Continued*

Class	Function and gene or fusion	Protein function; possible role in host interactions	Organism	Host or environment	Selection	Reference
	xylR	Xylose operon regulator (AraC family)	*L. monocytogenes*	Macrophage	DFI	295
	xylR	Xylose operon regulator (AraC family)	*E. chrysanthemi*	Spinach	*gfp-LA*	299
	srlD	Sorbitol-6-phosphate dehydrogenase	*P. multocida*	Mouse	*kan*	108
	yoxD	Ribitol dehydrogenase	*S. aureus*	Mouse	RIVET	152
	uxuA	Mannonate dehydratase	*H. influenzae*	Chinchilla	DFI	164
	ram2	α-L-Rhamnosidase	*L. plantarum*	Mouse	RIVET	24
	pbg10	6-Phospho-β-glucosidase	*L. plantarum*	Mouse	RIVET	24
	thgA1	Galactoside *O*-acetyltransferase	*L. plantarum*	Mouse	RIVET	24
	vca0242	Hexulose-6-phosphate synthase	*V. cholerae*	Mouse	RIVET	191
	glgA	Glycogen synthase	*R. solanacearum*	Tomato	*trpEG*	26
	glgB	1,4-α-Glucan branching enzyme	*V. cholerae*	Mouse	RIVET	191
	glgX	Glycogen operon protein	*R. solanacearum*	Tomato	*trpEG*	26
	malQ	4-α-Glucanotransferase	*V. cholerae*	Mouse	RIVET	191
	iviE	Exo-1,4-β-cellobiohydrolase	*S. gordonii*	Rabbit	*cat*	127
	iviH	Endo-1,3-β-glucanase	*S. gordonii*	Rabbit	*cat*	127
Amino acid synthesis						
Arginine biosynthesis						
	argA	***N-Acetyl glutamate synthase***	***V. cholerae***	**Mouse**	**RIVET**	**32**
	argF	Ornithine carbamoyl transferase	*H. influenzae*	Chinchilla	DFI	164
	argG	Argininosuccinate synthase	*R. solanacearum*	Tomato	*trpEG*	26
	argG	Argininosuccinate synthase	*L. plantarum*	Mouse	RIVET	24
	argH	Argininosuccinate lyase	*V. cholerae*	Mouse	RIVET	191
	carAB	**Carbamoylphosphate synthetase (arginine/pyrimidine nucleotide biosynthesis)**	***S. enterica***	**Mouse**	***purA***	**156**
	α-*carA*	Antisense transcript—carbamoylphosphate synthetase	*P. gingivalis*	Mouse	*tet*	298
Aromatic amino acid biosynthesis						
	aroQ	Chorismate mutase	*S. enterica*	Mouse	DFI	30
	trpD3	Anthranilate phosphoribosyl transferase (tryptophan biosynthesis)	*R. solanacearum*	Tomato	*trpEG*	26
	trpG	Anthranilate synthase β subunit (tryptophan biosynthesis)	*V. cholerae*	Mouse	RIVET	191
	trpR	Central regulator of tryptophan-related operons	*S. flexneri*	HeLa monolayer	*cat*	14
	pheA	Chorismate mutase-P/prephenate dehydratase (phenylalanine/tyrosine biosynthesis)	*E. chrysanthemi*	Spinach	*gfp-LA*	299
Cysteine biosynthesis						
	cysI	Sulfite reductase subunit	*V. cholerae*	Mouse	RIVET	32
	sseA	Thiosulfate sulfurtransferase	*M. tuberculosis*	Macrophages	DFI	275
Histidine biosynthesis						
	hisA	Phosphoribosylformimino-5-aminoimidazole carboxamide ribotide isomerase	*V. cholerae*	Mouse	RIVET	191
	hisB	Imidazole glycerol phosphate dehydratase	*P. aeruginosa*	Mouse	*purEK*	290
	hisB	Imidazole glycerol phosphate dehydratase	*H. influenzae*	Chinchilla	DFI	164
Branched amino acid biosynthesis						
	ilvA	Threonine deaminase (isoleucine biosynthesis)	*A. pleuropneumoniae*	Pig	*ribBAH*	76
	ilvA	Threonine deaminase (isoleucine biosynthesis)	*P. aeruginosa*	Mouse	*purEK*	290
	ilvI	Acetolactate synthase subunit	*A. pleuropneumoniae*	Pig	*ribBAH*	76
	ilvI	Acetolactate synthase subunit	*P. fluorescens*	Sugar beet	*dapB*	78
	ilvI	Acetolactate synthase subunit	*P. syringae*	*A. thaliana*	*hrcC*	16
Lysine biosynthesis						
	lysA	Diaminopimelate decarboxylase	*K. pneumoniae*	Mouse	*galU*	133
	lysA	Diaminopimelate decarboxylase	*S. flexneri*	Epithelial cells	DFI	233

Continued on following page

TABLE 2—*Continued*

Class	Function and gene or fusion	Protein function; possible role in host interactions	Organism	Host or environment	Selection	Reference
	dcdA	Diaminopimelate decarboxylase (LysA homologue)	*P. syringae*	*A. thaliana*	*hrcC*	16
	dapC	*N*-Succinyldiaminopimelate aminotransferase	*R. solanacearum*	Tomato	*trpEG*	26
	Methionine biosynthesis					
	metL	Aspartokinase/homoserine reductase (homoserine biosynthesis)	*Y. enterocolitica*	Mouse	*cat*	83
	50-55S3	Homoserine-*O*-acetyltransferase	*H. capsulatum*	Macrophage	*URA5*	226
	cysC	Homoserine-*O*-acetyltransferase	*H. capsulatum*	Mouse	*URA5*	226
	metR	Transcription regulator (LysR family)	*E. chrysanthemi*	Spinach	*gfp-LA*	299
	Proline biosynthesis					
	proC	Pyrroline-5-carboxylate reductase	*M. tuberculosis*	Macrophage	*inhA*	56
	proA	Glutamate-5-semialdehyde dehydrogenase	*L. plantarum*	Mouse	RIVET	24
	Threonine biosynthesis					
	thrC	Threonine synthase	*S. suis*	Pig	*erm*	254
	tdcA	Transcriptional activator of *tdc* operon	*K. pneumoniae*	Mouse	*galU*	133
	Glutamate/aspartate biosynthesis					
	gltB1	Glutamate synthase (large subunit)	*V. cholerae*	Mouse	RIVET	191
	gdhA	Glutamate dehydrogenase	*R. solanacearum*	Tomato	*trpEG*	26
	ansA	Cytoplasmic L-asparaginase I	*E. chrysanthemi*	Spinach	*gfp-LA*	299
Amino acid degradation						
gcvP		Glycine cleavage system (P protein)	*R. solanacearum*	Tomato	*trpEG*	26
gcvH1		Glycine cleavage system (H protein)	*P. syringae*	*A. thaliana*	*hrcC*	16
gcsH1		Glycine cleavage system (H protein)	*L. plantarum*	Mouse	RIVET	24
Nucleotide synthesis						
	De novo synthesis of pyrimidine nucleotides					
	pyrG	CTP synthase	*R. solanacearum*	Tomato	*trpEG*	26
	dut	Deoxyuridine triphosphatase	*M. tuberculosis*	Macrophage	*inhA*	56
	rsuA	16S rRNA pseudouridylate synthase	*L. plantarum*	Mouse	RIVET	24
	De novo synthesis of purine nucleotides					
	purF	Amidophosphoribosyltransferase	*R. solanacearum*	Tomato	*trpEG*	26
	purC	**Phosphoribosylaminoimidazole-Succinocarboxamide synthase**	***S. pneumoniae***	**Mouse**	**DFI**	**160**
	purE	Phosphoribosylaminoimidazole carboxylase subunit	*H. influenzae*	Chinchilla	DFI	164
	adk	Adenylate kinase	*L. plantarum*	Mouse	RIVET	24
	α-*ivi12*	Antisense transcript— phosphoribosylglycinamide formyltransferase	*P. gingivalis*	Mouse	*tet*	298
	Salvage pathways					
	upp	Uracil phosphoribosyltransferase	*E. chrysanthemi*	Spinach	*gfp-LA*	299
	Rsc0204	Thymidine/pyrimidine-nucleoside phosphorylase	*R. solanacearum*	Tomato	*trpEG*	26
	udp-1	Uridine phosphorylase	*V. cholerae*	Mouse	RIVET	191
	udk-dcd	Uridine kinase/dCTP deaminase	*P. multocida*	Mouse	*kan*	108
	xdhA	Xanthine dehydrogenase	*R. solanacearum*	Tomato	*trpEG*	26
	lp_0696	Cytosine/adenosine deaminase	*L. plantarum*	Mouse	RIVET	24
Peptide and protein synthesis						
	Nonribosomal peptide synthesis					
	P163	Peptide synthetase	*M. marinum*	Macrophage	DFI	11
	Rsp1419	Peptide synthetase	*R. solanacearum*	Tomato	*trpEG*	26
	Ribosomal synthesis					
	fusA2	Elongation factor G	*M. tuberculosis*	Macrophage	*inhA*	56
	EF-Tu	Elongation factor Tu	*L. monocytogenes*	Macrophage	DFI	295
	rrf	Ribosome recycling factor	*S. aureus*	Mouse	RIVET	152
	prfB-N	Peptide chain release factor 2 (N-terminal fragment)	*L. plantarum*	Mouse	RIVET	24
	prfB-C	Peptide chain release factor 2 (C-terminal fragment)	*L. plantarum*	Mouse	RIVET	24

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TABLE 2—*Continued*

Class	Function and gene or fusion	Protein function; possible role in host interactions	Organism	Host or environment	Selection	Reference
	α-*prfC*	Antisense transcript—peptide chain release factor 3	*R. solanacearum*	Tomato	*trpEG*	26
	dbpA	DEAD-type RNA helicase	*P. aeruginosa*	Mouse	*purEK*	290
	tgt	Queuine-tRNA ribosyltransferase	*Y. enterocolitica*	Mouse	*cat*	300
	α-*cii61*	Antisense transcript—16S rRNA gene	*P. stutzeri*	Rice	*dapB*	a
	Amino acid tRNA synthetases					
	alaS	Alanyl-tRNA synthetase	*R. solanacearum*	Tomato	*trpEG*	26
	argS	Arginyl-tRNA synthetase	*M. marinum*	Macrophage	DFI	11
	cysS	Cysteinyl-tRNA synthetase	*E. chrysanthemi*	Spinach	*gfp-LA*	299
	glnS	Glutaminyl-tRNA synthetase	*R. solanacearum*	Tomato	*trpEG*	26
	pheST	Phenylalanyl-tRNA synthetase	*S. enterica*	Mouse	*purA*	156
	tyrS	Tyrosyl-tRNA synthetase	*R. solanacearum*	Tomato	*trpEG*	26
	gatA	Glu-tRNA (gln) amidotransferase (subunit A)	*R. solanacearum*	Tomato	*trpEG*	26
	Protein folding					
	ppiA	Peptidyl prolyl *cis-trans* isomerase A	*K. pneumoniae*	Mouse	*galU*	133
	groEL$_5$	Chaperonine	*S. meliloti*	Alfalfa nodules	*bacA*	188
	dsbB	Disulfide oxidoreductase	*H. influenzae*	Chinchilla	DFI	164
	dsbD	**Thiol-disulfide interchange protein**	***P. multocida***	**Mouse**	***kan***	**108**
Protein degradation						
	pepN	Aminopeptidase N	*R. solanacearum*	Tomato	*trpEG*	26
	Rsp0196	Prolyl aminopeptidase	*R. solanacearum*	Tomato	*trpEG*	26
	ipx41	Carboxypeptidase	*P. syringae*	*A. thaliana*	*hrcC*	16
	Rsc1476	Carboxypeptidase	*R. solanacearum*	Tomato	*trpEG*	26
	pepD1	Dipeptidase	*L. plantarum*	Mouse	RIVET	24
	P238	Zinc metalloprotease	*M. marinum*	Macrophage	DFI	11
	ipc017	Zinc metalloprotease	*S. pneumoniae*	Mouse	DFI	160
	hreP	**Protease**	***Y. enterocolitica***	**Mouse**	***cat***	**300**
	IPC001	Serine protease	*S. pneumoniae*	Mouse	DFI	160
	Rsp0603	Serine protease	*R. solanacearum*	Tomato	*trpEG*	26
	Rsc3101	Serine protease	*R. solanacearum*	Tomato	*trpEG*	26
	α-*Rsc2654*	Antisense transcript—serine protease	*R. solanacearum*	Tomato	*trpEG*	26
	Rsc3101	Serine protease	*R. solanacearum*	Tomato	*trpEG*	26
	lon	ATP-dependent protease	*R. solanacearum*	Tomato	*trpEG*	26
	clpC	ATP-dependent protease Clp (ATPase subunit)	*L. plantarum*	Mouse	RIVET	24
Cofactor biosynthesis						
	Biotin biosynthesis					
	bioA	*S*-Adenosylmethionine-8-amino-7-oxononanoate aminotransferase	*S. flexneri*	Epithelial cells	DFI	233
	bioA	*S*-Adenosylmethionine-8-amino-7-oxononanoate aminotransferase	*V. cholerae*	Mouse	RIVET	191
	Rsc0082	*S*-Adenosylmethionine-8-amino-7-oxononanoate aminotransferase	*R. solanacearum*	Tomato	*trpEG*	26
	bioH	Biotin synthase	*Y. enterocolitica*	Mouse	*cat*	83
	birA2	Repressor of biotin ligase and biotin operon	*L. plantarum*	Mouse	RIVET	24
	Fe-S cluster biosynthesis					
	sufA	Fe-S cluster maturation protein	*S. flexneri*	Epithelial cells	DFI	233
	nifS	Cysteine desulfurase (synthesis nitrogenase metallocluster)	*S. meliloti*	Alfalfa nodules	*bacA*	188
	Nucleotide cofactor biosynthesis					
	nadE	NH_3-dependent NAD^+ synthetase	*P. gingivalis*	Mouse	*tet*	298
	Rsc2193	Nicotinate nucleotide adenylyltransferase	*R. solanacearum*	Tomato	*trpEG*	26
	ribB	3,4-Dihydroxy-2-butanone 4-phosphate synthase	*H. influenzae*	Chinchilla	DFI	164
	ribC2	Bifunctional riboflavin kinase and FMN adenylyltransferae	*L. plantarum*	Mouse	RIVET	24
	pncA	Pyrazinamidase/nicotinamidase	*E. chrysanthemi*	Spinach	*gfp-LA*	299
	Tetrapyrrole synthesis					
	hemA	Heme synthesis	*S. enterica*	Mouse	*purA*	98
	hemB	δ-Aminolevulinic acid dehydratase	*R. solanacearum*	Tomato	*trpEG*	26

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TABLE 2—*Continued*

Class	Function and gene or fusion	Protein function; possible role in host interactions	Organism	Host or environment	Selection	Reference
	hemD	Heme synthesis	*Y. enterocolitica*	Mouse	*cat*	300
	cii-11	Putative tetrapyrrole methylase (PA4422 homologue)	*P. stutzeri*	Rice	*dapB*	224
	Thiamine biosynthesis					
	thiE	Thiamine phosphate pyrophosphatase	*R. leguminosarum*	Pea nut	DFI	3
	thiF	Adenylation of ThiS	*V. cholerae*	Mouse	RIVET	191
	ipx45	Lipoprotein	*P. syringae*	*A. thaliana*	*hrcC*	16
	Biosynthesis of other cofactors					
	moeZ	Putative molybdopterin biosynthetic enzyme	*M. tuberculosis*	Macrophages	DFI	275
	nadB2	L-Aspartate oxidase (quinolinate biosynthesis)	*R. solanacearum*	Tomato	*trpEG*	26
	HI1647	Pyridoxine biosynthesis protein	*H. influenzae*	Chinchilla	DFI	164
	folB	Dihydroneopterin aldolase (folate biosynthesis)	*E. chrysanthemi*	Spinach	*gfp-LA*	299
	SPIV021	Flavodoxin	*S. pneumoniae*	Mouse	DFI	160
	Conversion of miscellaneous or unknown compounds					
	dhaT	1,3-Propanediol dehydrogenase	*L. plantarum*	Mouse	RIVET	24
	mdcA	Malonate decarboxylase	*P. fluorescens*	Sugar beet	*dapB*	78
	glcF	Glycolate oxidase (Fe-S subunit)	*R. solanacearum*	Tomato	*trpEG*	26
	gph	Phosphoglycolate phosphatase	*R. solanacearum*	Tomato	*trpEG*	26
	rhi-4	MorB-like reductase (complex N-compounds)	*P. fluorescens*	Sugar beet	*panB*	218
	adhE	Bifunctional alcohol and acetaldehyde dehydrogenase	*L. plantarum*	Mouse	RIVET	24
	vanR	Transcriptional regulator of GntR family (aromatic compounds)	*P. fluorescens*	Sugar beet	*dapB*	78
	pcaC	Carboxymuconolactone decarboxylase (aromatic compounds)	*P. stutzeri*	Rice	*dapB*	224
	gabD1	Succinate semialdehyde dehydrogenase (putrescine degradation)	*R. solanacearum*	Tomato	*trpEG*	26
	SPIV022	4-Oxalocrotonate tautomerase	*S. pneumoniae*	Mouse	DFI	160
	ipx 39	Haloacid dehalogenase-like hydrolase	*P. syringae*	*A. thaliana*	*hrcC*	16
	pup-28	Dioxygenase	*E. chrysanthemi*	Spinach	*gfp-LA*	299
	yiaK	Putative dehydrogenase	*P. multocida*	Mouse	*kan*	108
	rhi-74	Putative amidohydrolase (plant nitrilase-like)	*P. fluorescens*	Sugar beet	*dapB*	78
	39B6	MocC-like oxidoreductase (opine-like compounds)	*B. abortus*	Macrophage	DFI	62
	Energy metabolism					
	Rsc0087	NADH dehydrogenase	*R. solanacearum*	Tomato	*trpEG*	26
	nex8	NADH-ubiquinone oxidoreductase subunit	*S. meliloti*	Alfalfa nodules	*bacA*	188
	ivs-18	NADH oxidase	*S. suis*	Pig	*erm*	254
	Rsc1280	Transmembrane 4Fe-S ferredoxin	*R. solanacearum*	Tomato	*trpEG*	26
	α-*Rsc0329*	Antisense transcript—ferredoxin 2Fe-S	*R. solanacearum*	Tomato	*trpEG*	26
	atpD	ATP synthase subunit	*S. aureus*	Mouse	RIVET	152
	phaZ	PHB depolymerase	*R. solanacearum*	Tomato	*trpEG*	26
Class IV: genes involved in stress response and adaptation						
	Oxidative stress					
	Glutathione and thioredoxin metabolism					
	gsh	Glutathione synthetase	*Y. enterocolitica*	Mouse	*cat*	300
	Rsc2501	γ-Glutamyltranspeptidase	*R. solanacearum*	Tomato	*trpEG*	26
	lsfA	Glutathione peroxidase	*P. fluorescens*	Sugar beet	*panB*	218
	orf6-iviM	Glutathione reductase	*S. gordonii*	Rabbit	*cat*	127
	ydhD	Glutaredoxin	*Y. enterocolitica*	Mouse	*cat*	300
	trxC	Thioredoxin II	*V. cholerae*	Mouse	RIVET	191

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TABLE 2—*Continued*

Class	Function and gene or fusion	Protein function; possible role in host interactions	Organism	Host or environment	Selection	Reference
	ykmA	Glutathione peroxidase	*P. fluorescens*	Sugar beet	*panB*	218
	Peroxidases/catalases					
	bcp	Bacterioferritin co-migratory protein (peroxidase)	*R. solanacearum*	Tomato	*trpEG*	26
	bcp	Peroxidase	*P. stutzeri*	Rice	*dapB*	224
	Rsc2800	Peroxidase	*R. solanacearum*	Tomato	*trpEG*	26
	nex1	**Peroxiredoxin**	***S. meliloti***	**Alfalfa nodules**	***bacA***	**188**
	ohr	Organic hydroperoxide reductase	*A. pleuropneumoniae*	Pig	*ribBAH*	76
	catF	Catalase	*P. syringae*	*A. thaliana*	*hrcC*	16
	Other					
	Rsp1530	L-Ascorbate oxidase	*R. solanacearum*	Tomato	*trpEG*	26
	msrA	Peptide methionine sulfoxide reductase	*E. chrysanthemi*	Spinach	*gfp-LA*	299
	msrB	Peptide methionine sulfoxide reductase	*L. reuteri*	Mouse	*erm*	285
	orf4-iviK	Peptide methionine sulfoxide reductase	*S. gordonii*	Rabbit	*cat*	127
	vc1500	PqiA family protein	*V. cholerae*	Mouse	RIVET	191
	pqiB	Paraquat-inducible protein	*P. fluorescens*	Sugar beet	*dapB*	78
	oxyR	Regulator (LysR family)	*S. suis*	Pig	*erm*	254
	soxR	**Two-component response regulator**	***P. aeruginosa***	**Burned mouse**	***purEK***	**92**
	indA	Indigoidine biosynthesis protein IndA	*E. chrysanthemi*	Spinach	*gfp-LA*	299
	Acid tolerance					
	cadA	**Lysine decarboxylase; cadaverine synthesis**	***V. cholerae***	**Rabbit, mouse**	**RIVET**	**32, 174**
	cadC	Transcriptional regulator of cadaverine synthesis (OmpR family)	*S. enterica*	Mouse	*purA*	98
	speF	Ornithine decarboxylase (polyamine synthesis)	*P. multocida*	Mouse	*kan*	108
	spe2	*S*-Adenosylmethionine decarboxylase (polyamine synthesis)	*H. capsulatum*	Mouse	*URA5*	226
	ureH	Urease accessory protein	*H. influenzae*	Chinchilla	DFI	164
	Osmoregulation					
	mdoG	Synthesis of periplasmic β-glucans	*R. solanacearum*	Tomato	*trpEG*	26
	mdoH	**Synthesis of periplasmic β-glucans**	***Y. enterocolitica***	**Mouse**	***cat***	**300**
	ndvB	Cyclic β-(1,2) glucan synthase	*R. leguminosarum*	Peanut	DFI	3
	otsA	Trehalose synthetase	*S. enterica*	Mouse	*purA*	98
	Low oxygen					
	nrfE	**Formate-dependent nitrite reduction protein**	***P. multocida***	**Mouse**	***kan***	**19, 108**
	hemB	Porphobilinogen synthase	*S. flexneri*	HeLa monolayer	*cat*	14
	nirT	Respiratory nitrite reductase	*V. cholerae*	Mouse	RIVET	32
	Rsc2859	Putative formate dehydrogenase	*R. solanacearum*	Tomato	*trpEG*	26
	Detoxification					
	Metal ion efflux and homeostasis					
	cutC	Copper homeostasis protein	*E. chrysanthemi*	Spinach	*gfp-LA*	299
	copA	Copper transporting ATPase	*L. plantarum*	Mouse	RIVET	24
	copA	Copper transporting ATPase	*S. aureus*	Mouse	RIVET	152
	copRS	Two-component response regulator of copper resistance	*P. fluorescens*	Sugar beet	*panB*	218
	ragC	Cation efflux protein	*P. fluorescens*	Sugar beet	*panB*	218
	lp_3288	Cation efflux protein	*L. plantarum*	Mouse	RIVET	24
	Rsp1597	Putative cation efflux pump	*R. solanacearum*	Tomato	*trpEG*	26
	czcS	Two-component sensor kinase for regulation of Zn, Co, Cd resistance	*R. solanacearum*	Tomato	*trpEG*	26
	iviX	Heavy metal transport	*S. enterica*	Mouse	*cat*	98
	ipx33	Metal transporting P-type ATPase	*P. syringae*	*A. thaliana*	*hrcC*	16
	Antibiotic resistance					
	Rsc1323	Multidrug resistance protein	*R. solanacearum*	Tomato	*trpEG*	26
	P155	Drug efflux pump	*M. marinum*	Macrophage	DFI	11
	lp_3303	Multidrug transport protein	*L. plantarum*	Mouse	RIVET	24

Continued on following page

TABLE 2—*Continued*

Class	Function and gene or fusion	Protein function; possible role in host interactions	Organism	Host or environment	Selection	Reference
	acrA	Periplasmic component of RND-type efflux pump	*R. solanacearum*	Tomato	*trpEG*	26
	acrF	Transmembrane protein of RND-type efflux pump	*P. fluorescens*	Sugar beet	*dapB*	78
	acrR	Transcriptional regulator (TetR)	*Y. enterocolitica*	Mouse	*cat*	300
	rosA	Transmembrane protein (fosmidomycin resistance)	*P. fluorescens*	Sugar beet	*panB*	218
	α-*Rsc3205*	Antisense transcript—transmembrane protein	*R. solanacearum*	Tomato	*trpEG*	26
	aph	Aminoglycoside 3′ phosphotransferase	*M. tuberculosis*	Macrophage	*inhA*	56
	ydhC	Drug resistance protein	*Y. ruckeri*	Fish	*cat*	65
	pmrB	**Regulator (polymyxin resistance)**	***S. enterica***	**Mouse**	***cat***	**90, 98**
	Methylglyoxal detoxification					
	ycbL	Glyoxylase II enzym family	*P. fluorescens*	Sugar beet	*panB*	218
	glxI	Lactoylglutathione lyase	*B. abortus*	Macrophage	DFI	62
	Bacteriocin autoimmunity					
	plnI	Bacteriocin (plantaricin) immunity protein	*L. plantarum*	Mouse	RIVET	24
	Other stress-related proteins					
	cspD	Cold shock-like protein	*H. influenzae*	Chinchilla	DFI	164
	hslU	Stress-inducible protease (ATPase component)	*S. enterica*	Macrophages	DFI	279
	hslU	Stress-inducible protease (ATPase component)	*R. solanacearum*	Tomato	*trpEG*	26
	grpE	Heat shock protein 24	*R. solanacearum*	Tomato	*trpEG*	26
	yhbH	RpoN modulator protein; nutritional adaptation	*P. stutzeri*	Rice	*dapB*	224
	ynaF	Universal stress protein	*E. chrysanthemi*	Spinach	*gfp-LA*	299
	ndk	Nucleoside diphosphate kinase (alarmone synthesis)	*S. enterica*	Mouse	*purA*	98
	dinF	DNA damage-inducible transmembrane protein (SOS response)	*R. solanacearum*	Tomato	*trpEG*	26
Class V: genes involved in regulation						
	Two-component regulatory systems					
	vsrB	Regulator	*R. solanacearum*	Tomato	*trpEG*	26
	vieB	**Regulator**	***V. cholerae***	**Mouse**	**RIVET**	**32**
	phoP	Regulator	*S. enterica*	Mouse	*purA*	98
	ivs-25	Regulator	*S. suis*	Pig	*erm*	254
	pehR	Regulator	*R. solanacearum*	Tomato	*trpEG*	26
	sapR	Regulator	*S. suis*	Pig	*erm*	254
	gacA	Regulator	*Y. enterocolitica*	Mouse	*cat*	83
	Rsc1597	Regulator (LuxR-like)	*R. solanacearum*	Tomato	*trpEG*	26
	vsrD	Regulator (LuxR-like)	*R. solanacearum*	Tomato	*trpEG*	26
	ipx48	Sensor	*P. syringae*	*A. thaliana*	*hrcC*	16
	ipx49	Sensor	*P. syringae*	*A. thaliana*	*hrcC*	16
	vc0622	Sensor	*V. cholerae*	Mouse	RIVET	191
	α-*phoR*	Antisense transcript—sensor (virulence)	*L. monocyto-genes*	Mouse	*hly*	55
	Transcriptional regulators					
	argR	Repressor (AraC family)	*H. influenzae*	Chinchilla	DFI	164
	iviG	Regulator (AraC family)	*S. gordonii*	Rabbit	*cat*	127
	lp_3646	Regulator (AraC family)	*L. plantarum*	Mouse	RIVET	24
	glpR	Repressor (DeoR family)	*H. influenzae*	Chinchilla	DFI	164
	cpsY	Regulator (LysR family)	*S. suis*	Pig	*erm*	254
	Rsc2094	Regulator (LysR family)	*R. solanacearum*	Tomato	*trpEG*	26
	Rsp1574	Regulator (LysR family)	*R. solanacearum*	Tomato	*trpEG*	26
	yeiE	**Regulator (LysR family)**	***Y. enterocolitica***	**Mouse**	***cat***	**300**
	tfdT	Regulator (LysR family)	*P. aeruginosa*	Mouse	*purEK*	290
	Rsp1644	Regulator (MarR family)	*R. solanacearum*	Tomato	*trpEG*	26
	srpS	Regulator (Crp family)	*E. chrysanthemi*	Spinach	*gfp-LA*	299
	pup-24	Regulator (Cro/CI family)	*E. chrysanthemi*	Spinach	*gfp-LA*	299

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TABLE 2—*Continued*

Class	Function and gene or fusion	Protein function; possible role in host interactions	Organism	Host or environment	Selection	Reference
	crp	CRP regulator	*E. chrysanthemi*	Spinach	*gfp-LA*	299
	relB	RelB protein	*E. chrysanthemi*	Spinach	*gfp-LA*	299
	nex7	Regulator (TspO/MBR family)	*S. meliloti*	Alfalfa nodules	*bacA*	188
	rhi-1	Putative repressor (DnrO-like)	*P. fluorescens*	Sugar beet	*panB*	218
	Rv0549c	Putative regulator	*M. tuberculosis*	Macrophage	*inhA*	56
	orf1-iviB	Regulator (Rgg-like)	*S. gordonii*	Rabbit	*cat*	127
	Rsc0280	Regulator (MerR family)	*R. solanacearum*	Tomato	*trpEG*	26
	Rsc1997	Regulator (GntR family)	*R. solanacearum*	Tomato	*trpEG*	26
	Serine/threonine protein kinases					
	iviJ		*S. gordonii*	Rabbit	*cat*	127
	ppkA		***P. aeruginosa***	**Mouse**	***purEK***	**288,290**
	pkn2		*L. plantarum*	Mouse	RIVET	24
	α-*lu1*	Antisense transcript	*H. capsulatum*	Mouse	*URA5*	226
	Transcription factors					
	greA	Transcription elongation factor	*R. solanacearum*	Tomato	*trpEG*	26
	hrpA	ATP-dependent RNA helicase	*R. solanacearum*	Tomato	*trpEG*	26
	infC	Initiation factor 3	*M. tuberculosis*	Macrophage	*inhA*	56
	rpoS	Sigma factor σ^{54}	*R. solanacearum*	Tomato	*trpEG*	26
	Rsp1668	σ^{54}-interacting protein	*R. solanacearum*	Tomato	*trpEG*	26
	himA	IHF subunit	*S. enterica*	Macrophages	DFI	279
	himA	**IHF subunit**	***S. enterica***	**Macrophages**	***purA***	**156**
	ihfB	IHF subunit	*H. influenzae*	Chinchilla	DFI	164
	nusG	Antitermination and rho-dependent termination	*A. pleuropneumoniae*	Pig	*ribBAH*	76
	bglG4	Antitermination	*L. plantarum*	Mouse	RIVET	24
	map 25	WhiB-like putative transcription factor	*M. marinum*	Macrophage	DFI	221
	Other					
	hfq	Global regulator	*P. stutzeri*	Rice	*dapB*	224
	hflX	Located in *mutL-hfq* superoperon	*Y. enterocolitica*	Mouse	*cat*	300
	ack-pta	Acetate kinase/phosphotransacetylase	*P. multocida*	Mouse	*kan*	108
	pta	Phosphotransacetylase	*P. stutzeri*	Rice	*dapB*	224
	agrA	Accessory gene regulator; virulence regulator	*S. aureus*	Mouse	RIVET	152
	aldR	Inhibitor of protein synthesis (cleavage of mRNA)	*S. suis*	Pig	*erm*	254
	cpdP	3′-5′ cAMP phosphodiesterase	*Y. enterocolitica*	Mouse	*cat*	300
	vc0130	c-di-GMP metabolism	*V. cholerae*	Mouse	RIVET	191
	pup-44	Zn-finger-containing protein	*E. chrysanthemi*	Spinach	*gfp-LA*	299
Class VI: genes involved in cell envelope structure and modification						
	Peptidoglycan					
	sltY	**Lytic transglycosylase**	***S. flexneri***	**HeLa monolayer**	***cat***	**14**
	ipx10	**Lytic transglycosylase**	***P. syringae***	***A. thaliana***	***hrcC***	**16**
	mtgA	Transglycosylase	*B. abortus*	Macrophage	DFI	62
	vc2487	Transglycosylase	*V. cholerae*	Mouse	RIVET	191
	pup-36	Membrane protein with C-terminal transglycosylase domain	*E. chrysanthemi*	Spinach	*gfp-LA*	299
	yvyH	*N*-Acetyl glucosamine epimerase	*L. monocytogenes*	Mouse	*hly*	55
	murC	UDP-*N*-acetylmuramoyl-L-alanine synthetase	*S. aureus*	Mouse	RIVET	152
	atl	*N*-Acetyl-muramoyl-L-alanine amidase	*S. aureus*	Mouse	RIVET	152
	Rv3717	*N*-Acetyl-muramoyl-L-alanine amidase	*M. tuberculosis*	Macrophage	*inhA*	56
	ampG	Muropeptide transporter	*P. syringae*	*A. thaliana*	*hrcC*	16
	pup-33	Periplasmic murein peptide-binding protein of ABC transporter	*E. chrysanthemi*	Spinach	*gfp-LA*	299
	pbp2	Penicilline-binding protein 2	*S. aureus*	Mouse	RIVET	152
	yrbH	Putative sugar isomerase	*S. flexneri*	HeLa monolayer	*cat*	14
	LPS/EPS biosynthesis					
	orf3-iviF	ADP-L-glycero-D-mannoheptose-6-epimerase	*S. gordonii*	Rabbit	*cat*	127

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TABLE 2—*Continued*

Class	Function and gene or fusion	Protein function; possible role in host interactions	Organism	Host or environment	Selection	Reference
	rffG	dTDP-glucose 4,6-dehydratase	*Y. enterocolitica*	Mouse	*cat*	83
	manB	Phosphomannomutase	*Y. enterocolitica*	Mouse	*cat*	83
	mrp	Putative ATPase	*A. pleuropneumoniae*	Pig	*ribBAH*	76
	rfb	Lipopolysaccharide synthesis	*S. enterica*	Mouse	*purA*	156
	algA	Alginate biosynthesis	*P. syringae*	*A. thaliana*	*hrcC*	16
	Adhesion/invasion					
	hag2	Hemagglutinin	*P. aeruginosa*	Mouse, rat	*purA*	93
	hagB	Hemagglutinin	*P. gingivalis*	Mouse	*tet*	141
	hagC	Hemagglutinin	*P. gingivalis*	Mouse	*tet*	141
	iviIV	Putative hemagglutinin	*Y. ruckeri*	Fish	*cat*	65
	iviVI-A	Tia-like protein	*S. enterica*	Mouse	*cat*	98
	iviVI-B	PfEMP1-like protein	*S. enterica*	Mouse	*cat*	98
	flpA	Fibronectin/fibrinogen-binding protein	*S. suis*	Pig	*erm*	254
	nex18	**Putative fasciclin-like adhesion molecule**	***S. meliloti***	**Alfalfa nodules**	***bacA***	**188**
	iviXIII	TadD-like protein	*Y. ruckeri*	Fish	*cat*	65
	tcpA	Pilin subunit of toxin-coregulated pilus	*V. cholerae*	Mouse	RIVET	139
	Outer membrane protein (gram-negative bacteria)					
	lolA	Outer membrane lipoprotein carrier protein	*H. influenzae*	Chinchilla	DFI	164
	pcp	**Lipoprotein**	***P. multocida***	**Mouse**	***kan***	**108**
	lppM	Lipoprotein	*M. tuberculosis*	Macrophage	*inhA*	56
	lppB	Lipoprotein	*H. influenzae*	Chinchilla	DFI	164
	Rsc1627	Lipoprotein	*R. solanacearum*	Tomato	*trpEG*	26
	sif15	**Outer membrane protein**	***Y. enterocolitica***	**Mouse**	***cat***	**83, 300**
	ompW	Outer membrane protein	*R. solanacearum*	Tomato	*trpEG*	26
	pup-38	Outer membrane protein	*E. chrysanthemi*	Spinach	*gfp-LA*	299
	wssE	**Cellulose synthase subunit**	***P. fluorescens***	**Sugar beet**	***dapB***	**78**
	vca1008	**Porin**	***V. cholerae***	**Mouse**	**RIVET**	**32, 192**
	oprD2	Porin	*P. putida*	*P. parasitica*	*pyrB*	140
	α-*oprE*	Antisense transcript—porin	*P. aeruginosa*	Mouse	*purEK*	290
	Cell surface proteins (gram-positive bacteria)					
	lp_0800		*L. plantarum*	Mouse	RIVET	24
	lp_2940		*L. plantarum*	Mouse	RIVET	24
	lp_1403		*L. plantarum*	Mouse	RIVET	24
	lp_0141		*L. plantarum*	Mouse	RIVET	24
Class VII: genes involved in virulence and secretion						
	Secretion					
	SRP pathway					
	tig	Trigger factor	*P. fluorescens*	Sugar beet	*panB*	218
	General secretory pathway					
	ftsY	Docking protein	*P. aeruginosa*	Mouse, rat	*purA*	93
	secA	Translocation ATPase	*S. meliloti*	Alfalfa nodules	*bacA*	188
	secE	Cytoplasmic membrane protein	*A. pleuropneumoniae*	Pig	*ribBAH*	76
	outFG	Component of Out system	*E. chrysanthemi*	Spinach	*gfp-LA*	299
	gspK	Type II secretory protein	*R. solanacearum*	Tomato	*trpEG*	26
	spsAB	Signal peptidase	*S. aureus*	Mouse	RIVET	152
	orf104	Signal peptidase	*L. monocytogenes*	Mouse	*hly*	55
	α-*cg316*	Antisense transcript—signal peptidase	*L. monocytogenes*	Mouse	*hly*	55
	TTSS structural components					
	ssaH		*S. enterica*	Macrophages	DFI	279
	hrcC		*R. solanacearum*	Tomato	*trpEG*	26
	hrpK		*P. syringae*	*A. thaliana*	*hrcC*	16

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TABLE 2—*Continued*

Class	Function and gene or fusion	Protein function; possible role in host interactions	Organism	Host or environment	Selection	Reference
	hrpJ		*P. syringae*	*A. thaliana*	*hrcC*	16
	hrpG		*P. syringae*	*A. thaliana*	*hrcC*	16
	hrcQb		*P. syringae*	*A. thaliana*	*hrcC*	16
	hrpA		*P. syringae*	*A. thaliana*	*hrcC*	16
	hrpA		*E. chrysanthemi*	Spinach	*gfp-LA*	299
	hrpB		***E. chrysanthemi***	**Spinach**	***gfp-LA***	**299**
	hrcC		*P. fluorescens*	Sugar beet	*panB*	218
	TTSS effector proteins					
	pipB	Glycolipid biosynthesis	*S. enterica*	Mouse	DFI	30
	sifA	Essential virulence factor	*S. enterica*	Mouse	DFI	30
	avrPphD		*P. syringae*	*A. thaliana*	*hrcC*	16
	avrPpiB		*P. syringae*	*A. thaliana*	*hrcC*	16
	virPphA		*P. syringae*	*A. thaliana*	*hrcC*	16
	dspA		*E. chrysanthemi*	Spinach	*gfp-LA*	299
	dspE		*E. chrysanthemi*	Spinach	*gfp-LA*	299
	TTSS regulatory proteins					
	ptrA	Inhibitor of transcriptional activator ExsA	*P. aeruginosa*	Burned mouse	*purEK*	91, 92
	Virulence factors					
	yhdP	**Hemolysin-like protein (erythrocyte lysis)**	***L. monocytogenes***	**Macrophage**	**DFI**	**295**
	shlB	Hemolysin activator protein	*Y. ruckeri*	Fish	*cat*	65
	plcA	Phosphatidylinositol phospholipase C	*L. monocytogenes*	Mouse	*hly*	55
	ispA	Hemolysin A homologue	*L. plantarum*	Mouse	RIVET	24
	ctxA	Catalytical subunit of CT (toxine) synthesis	*V. cholerae*	Mouse	RIVET	139
	spvB	**Virulence factor**	***S. enterica***	**Mouse**	***cat***	**98**
	mig-5	**Virulence plasmid lipoprotein**	***S. enterica***	**Macrophages**	**DFI**	**279**
	α-*mviM*	Antisense transcript—virulence factor	*L. monocytogenes*	Mouse	*hly*	55
	actA	Actin recruitement and polymerisation protein	*L. monocytogenes*	Macrophage	DFI	295
	exc	Plasmid extrusion protein (TraT-like)	*S. enterica*	Macrophages	DFI	279
	ivi11	**Immunoreactive antigen**	***P. gingivalis***	**Mouse**	***tet***	**298**
	lpxA	Acyltransferase	*Y. enterocolitica*	Mouse	*cat*	300
	plyD	Pectin lyase	*P. syringae*	*A. thaliana*	*hrcC*	16
	pme	Membrane-bound pectinesterase	*R. solanacearum*	Tomato	*trpEG*	26
	ogl	Oligogalacturonate lyase	*E. chrysanthemi*	Spinach	*gfp-LA*	299
	syrE	Syringomycin synthetase (phytotoxin production)	*P. syringae*	*A. thaliana*	*hrcC*	16
	cfl-cfa	Coronatine biosynthesis (phytotoxin production)	*P. syringae*	*A. thaliana*	*hrcC*	16
	pup-29	MsgA-like virulence protein	*E. chrysanthemi*	Spinach	*gfp-LA*	299
	ivs-21	Extracellular protein	*S. suis*	Pig	*erm*	254
	SAP2	Secreted protease	*C. albicans*	Mouse	RIVET	262
	Rv2224c	Secreted protease	*M. tuberculosis*	Macrophage	*inhA*	56
	map 24	**PE-PGRS virulence protein**	***M. marinum***	**Macrophage**	**DFI**	**221**
	map 85	PE-PGRS protein	*M. marinum*	Macrophage	DFI	221
	vc2621	Extracellular nuclease-related protein	*V. cholerae*	Mouse	RIVET	191
	iaaM	Tryptophan 2-monooxygenase (auxin biosynthesis)	*E. chrysanthemi*	Spinach	*gfp-LA*	299
	vc1619.1	Putative RTX toxin	*V. cholerae*	Mouse	RIVET	191
	Regulation					
	bvrA	Antiterminator of *bvrBC,* which encodes β-glucoside-specific permease	*L. monocytogenes*	Macrophage	DFI	295
	vacB	RNA processing	*S. enterica*	Mouse	*purA*	98
	vacC	RNA processing	*S. enterica*	Mouse	*purA*	98
	vacC	RNA processing	*Y. enterocolitica*	Mouse	*cat*	300
	chvD	Two-component response regulator	*S. enterica*	Mouse	*purA*	98
	virB	Regulator virulence genes (ParB-type)	*S. flexneri*	HeLa monolayer	*cat*	14
Class VIII: genes involved in nucleic acid metabolism						
	DNA synthesis					
	iviC	Subunit of DNA polymerase III	*S. gordonii*	Rabbit	*cat*	127

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TABLE 2—*Continued*

Class	Function and gene or fusion	Protein function; possible role in host interactions	Organism	Host or environment	Selection	Reference
	dnaN	Subunit of DNA polymerase III	*R. solanacearum*	Tomato	*trpEG*	26
	dnaX	Subunit of DNA polymerase III	*L. plantarum*	Mouse	RIVET	24
	traI	Relaxase	*Y. ruckeri*	Fish	*cat*	65
	DNA topology					
	gyrA	Subunit of DNA gyrase	*K. pneumoniae*	Mouse	*galU*	133
	topB	DNA topoisomerase III	*L. monocytogenes*	Mouse	*hly*	77
	RTI004	DNA topoisomerase IV	*S. pneumoniae*	Mouse	DFI	160
	rhlB	Helicase	*Y. enterocolitica*	Mouse	*cat*	83
	helA	**Helicase**	***P. fluorescens***	**Sugar beet**	***dapB***	**302**
	helB	Helicase	*P. fluorescens*	Sugar beet	*dapB*	302
	helC	Helicase	*P. fluorescens*	Sugar beet	*dapB*	302
	DNA repair					
	alkA	**Methyladenine DNA glycosylase**	***S. flexneri***	**HeLa monolayer**	***asd***	**244**
	udg	Uracil DNA glycosylase	*L. monocytogenes*	Macrophage	DFI	295
	mutL	DNA mismatch repair protein	*Y. enterocolitica*	Mouse	*cat*	300
	uvrA	Subunit exonuclease ABC	*R. solanacearum*	Tomato	*trpEG*	26
	uvrB	Subunit exonuclease ABC	*P. gingivalis*	Mouse	*tet*	298
	uvrC	Subunit exonuclease ABC	*M. tuberculosis*	Macrophage	*inhA*	56
	recB	Subunit of exodeoxyribonuclease	*Y. enterocolitica*	Mouse	*cat*	300
	recD	**Subunit of exodeoxyribonuclease**	***S. enterica***	**Mouse**	***cat***	**98**
	recG	DNA helicase	*H. influenzae*	Chinchilla	DFI	164
	DNA methylation					
	met	Methyltransferase	*L. monocytogenes*	Mouse	*hly*	77
	cl 136	Methyltransferase	*L. monocytogenes*	Macrophage	DFI	295
	mtpS	DNA methylase	*Y. enterocolitica*	Mouse	*cat*	300
	RNA degradation					
	orn	Oligoribonuclease	*P. fluorescens*	Sugar beet	*dapB*	303
	rbn	RNase BN	*V. cholerae*	Mouse	RIVET	191
	Cell division					
	SPSpoJ	Chromosome segregation protein	*S. pneumoniae*	Mouse	DFI	160
	ispZ	Septation protein	*H. influenzae*	Chinchilla	DFI	164
	mukF	Chromosome partition protein	*H. influenzae*	Chinchilla	DFI	164
	crcB	Chromosome condensation protein	*E. chrysanthemi*	Spinach	*gfp-LA*	299
Class IX: genes involved in transposition and site-specific recombination						
	Mobile DNA elements					
	IS600	Transposase (family 8)	*S. flexneri*	Epithelial cells	DFI	233
	tnpA	Transposase (family 11; IS*4*-like)	*P. syringae*	*A. thaliana*	*hrcC*	16
	ivs-8	Transposase (family 11)	*S. suis*	Pig	*erm*	254
	tnpA	Transposase (family 17; IS*200*-like)	*E. chrysanthemi*	Spinach	*gfp-LA*	299
	tnp	Transposase (Tn5-like)	*Y. enterocolitica*	Mouse	*cat*	300
	ivs-1	Putative transposase	*S. suis*	Pig	*erm*	254
	iviA	Putative transposase	*S. gordonii*	Rabbit	*cat*	127
	issfl4	Insertion sequence	*S. flexneri*	HeLa monolayer	*cat*	14
	tnpF	IS*2*-like	*S. flexneri*	HeLa monolayer	*cat*	14
	ivi5	IS*195*-like	*P. gingivalis*	Mouse	*tet*	298
	gipA	**IS*891*-like**	***S. enterica***	**Mouse**	***purA***	**264**
	vgrG	Vgr-like protein (located on multicopy genetic elements)	*E. chrysanthemi*	Spinach	*gfp-LA*	299
	vgrG	Vgr-like protein (located on multicopy genetic elements)	*B. pseudomallei*	Macrophage	*cat*	b
	Integrases/recombinases					
	Rsp1303	Integrase	*R. solanacearum*	Tomato	*trpEG*	26
	rinA	Integrase gene activator	*S. aureus*	Mouse	RIVET	152
	redF	Resolvase	*P. aeruginosa*	Mouse	*purEK*	290
	xerD	Site-specific DNA recombinase	*P. syringae*	Bean	*metXW*	159
	xerD	Site-specific DNA recombinase	*B. abortus*	Macrophage	DFI	62
	xerD	Site-specific DNA recombinase	*V. cholerae*	Mouse	RIVET	191
	codV	Integrase/recombinase	*L. plantarum*	Mouse	RIVET	24

[a] Overview of promoters of genes or operons that are upregulated in microorganisms during their interaction with a eukaryotic host, isolated with the promoter-trapping techniques IVET and DFI. The (predicted) cellular function of the corresponding microbial gene product(s) in the respective environmental niche is indicated. For each IVET study, the selection strategy is specified. Most assignments of genes and gene product functions are based on similarity to known genes as described in the original papers. Some of these assignments were further verified or updated through homology searches with available gene sequences. Genes subsequently shown to be required for interaction with a host are indicated in bold. Abbreviations: ABC, ATP-binding cassette; CRP, cAMP receptor protein; *gfp-LA*, GFP-based IVET leaf array; IHF, integration host factor; MCP, methyl-accepting chemotaxis protein; PHB, polyhydroxybutyrate; PTS, phosphotransferase system; SRP, signal recognition particle; TRAP-T, tripartite ATP-independent periplasmic transporter; TTSS, type III secretion system. a, H. Rediers, unpublished results; b, M. S. Thomas, unpublished results.

on homology or the presence of characteristic domains in the putative gene products. However, these assignments are valuable to group the large number of promoter-trapped genes in functional categories relevant to bacterial physiology. Such classification allows identification of commonalities as well as differences in gene expression patterns for these widely different experimental systems, involving phylogenetically diverse pathogenic and nonpathogenic microorganisms residing in diverse complex environments, in particular plant or animal hosts. In the following, these functional classes are discussed in more detail, with emphasis on new insights that resulted from IVET or DFI studies.

Genes Involved in Chemotaxis and Motility

Table 2 shows that several chemotaxis- and motility-related genes are upregulated in various plant- and animal-colonizing bacteria during interaction with their host. For instance, the *fliF* gene is expressed in *Streptococcus suis* infecting piglets (254) and in *Pseudomonas fluorescens* colonizing sugar beet rhizosphere (78). Multiple copies (≈25) of the FliF protein are assembled into the cytoplasmic membrane-embedded MS-ring, which constitutes the core of the flagellar motor (268). The isolation of these *fliF* genes indicates that the flagellar machinery is important for gram-positive as well as for gram-negative bacteria to establish interactions with both animal and plant hosts. This conclusion from IVET studies is in line with the results from other studies. For efficient colonization of tomato roots by nonpathogenic *Pseudomonas fluorescens*, flagellum-driven chemotaxis is required (48). In several other cases where competition between several bacterial species exists, flagellum-mediated motility is shown to provide a specific advantage for bacteria (181). For instance, for *Vibrio cholerae*, it was shown that mutants with knockouts in a flagellar subunit gene (*flaA*) or in genes encoding flagellar motor proteins (*motAB* and *motY*) are severely affected in colonization of the mouse small intestine (137).

Besides providing motility, flagella are important for bacterial attachment to surfaces and are thus generally considered important virulence factors (45, 181). Therefore, it is not surprising that the flagellar subunit is recognized by the innate immune system in organisms as diverse as plants (306) and mammals (256). This implies that for a successful host infection to occur, a bacterial pathogen might need to suppress flagellar assembly after the initial interaction stage. This requires strictly coordinated temporal gene expression during the different stages of interaction. Antisense transcripts of target genes may be involved in this. A putative antisense α-*flaA* was identified in *Vibrio cholerae* by RIVET (32). Likewise, a putative antisense transcript of *fliM*, encoding the flagellar C-ring, is upregulated in *Pseudomonas stutzeri* A15 during rice root colonization (H. Rediers, unpublished data). It is known that some bacteria adapt their flagellation pattern in response to the environmental conditions they encounter (reviewed in references 73 and 170).

The apparent temporal expression/repression of flagellin synthesis may be coordinated with the expression/repression of the cognate chemosensory machinery (Che system). A *cheY*-containing transcriptional fusion was isolated by an IVET screening in *Pseudomonas aeruginosa* infecting mice (290) and *Ralstonia solanacearum* infecting tomato plants (26). CheY is the response regulator protein that, in its phosphorylated form, interacts with the switch machinery of the flagellar motor to change the direction or speed of rotation (269). In addition to the temporal regulation of flagellin genes, chemotaxis may be fine-tuned throughout the infection process by differential expression of methyl-accepting chemotaxis protein subsets and multiple motility-linked chemosensory systems that are present in many bacteria, such as *Vibrio* spp. (169), *Pseudomonas* spp. (66), and *Rhodobacter* spp. (163). In two IVET studies, putative antisense transcripts of chemotaxis-related genes, encoding both sensory (α-*mcp*) and signal transduction (α-*cheV*) proteins were identified in rice-colonizing *Pseudomonas stutzeri* (224) and *Vibrio cholerae* infecting mice, respectively (32). CheV encodes a chimeric protein containing a CheY-homologous domain. It has been shown that a *Vibrio cholerae cheV* mutant colonizes mice better than the wild type (32). This is in agreement with the reciprocal regulation of motility and virulence genes in *Vibrio cholerae* (80). Downregulating chemotaxis genes might increase infection efficiency by favoring the formation and maintenance of microcolonies (175).

IVET also revealed that a gene involved in type IV pilus biogenesis is upregulated in *Ralstonia solanacearum* during tomato infection (26). Type IV pili enable twitching motility, a pilus-based form of translocation used by pathogens to spread over the host tissue surface, and are therefore recognized as important virulence factors for a wide range of plant and animal pathogens. In addition, type IV pili are important for the formation of biofilms and fruiting bodies (165).

Genes Involved in Nutrient Scavenging

Homeostasis of iron and other metal ions. In numerous promoter-trapping studies, irrespective of the chosen selection strategy, genes involved in siderophore-dependent and siderophore-independent iron uptake as well as other genes involved in metal ion scavenging were found to be induced in both gram-negative and gram-positive bacteria (Table 2). Siderophores are secreted to bind Fe(III) with high affinity (reviewed in reference 222). Genes involved in the biogenesis of different types of siderophores (aerobactin, enterobactin, pyoverdin, ruckerbactin, and yersiniabactin) display elevated expression levels during the life of different γ-proteobacterial species in a plant or animal host environment.

After iron chelation, ferrisiderophores are captured at the cell surface by specific high-affinity siderophore receptors. Consistent with the induction of siderophore biosynthesis, several genes encoding such receptors have been identified by IVET or DFI. The ferric hydroxamate receptor, encoded by *fhuA*, is induced in both *Salmonella enterica* serovar Typhimurium infecting mice (98) and *Shigella flexneri* infecting monolayers of human epithelial cells (233). In *Porphyromonas gingivalis*, a putative siderophore receptor encoded by *ivi10* is specifically induced during infection of mice. Wu et al. (298) demonstrated that a *Porphyromonas gingivalis ivi10* knockout mutant is outcompeted by the wild type during survival in the host and displays a reduced ability to cause infection. The active translocation of ferrisiderophores is Ton dependent and is driven by the proton motive force. Following TonB-dependent translocation, ferrisiderophores are finally transported

into the cytoplasm by an ATP-binding cassette (ABC) transporter. Genes encoding the RupDGC transporter as well as other genes involved in ruckerbactin-mediated iron uptake (synthesis of siderophore and receptor and TonB-dependent translocation) were identified by IVET in *Yersinia ruckeri* infecting fish (65).

Upon infection, several pathogenic bacteria display upregulation of genes encoding siderophore-independent iron uptake systems, such as the *Salmonella enterica* serovar Typhimurium SitABCD transporter (305). Application of IVET revealed that the *sitABCD* operon is specifically induced during infection of mice. It was subsequently demonstrated that a *Salmonella enterica* serovar Typhimurium *sit* mutant is severely attenuated in infection of mice (114). The *sitABCD* operon encodes an ABC transport system that mediates iron and probably also manganese uptake (124). *Erwinia chrysanthemi yfeA*, which encodes a component of a Sit-homologous transport system, is upregulated during plant infection (299). Other promoter trap studies demonstrated that *sitA* and *sitC* homologues are specifically expressed in *Shigella flexneri* (233) and *Yersinia enterocolitica* (83), respectively, during infection of mice. Although a *sitA* mutation does not affect plaque formation by *Shigella flexneri* on monolayers of human intestinal epithelial cells, a *sitA* mutation in combination with other iron acquisition mutations shows additive effects in these plaque assays (234).

Some bacteria possess mechanisms for the uptake of siderophores that are produced by other species or for uptake of iron-containing host proteins such as transferrin or heme (232, 251). IVET revealed the upregulation of genes involved in hemin uptake (*hmuS*, *hmuT*, and *hmuU*) in spinach-infecting *Erwinia chrysanthemi* (299). The *Burkholderia pseudomallei hemT* gene, encoding a periplasmic hemin binding protein, was also shown to be induced during macrophage infection (M. S. Thomas, personal communication).

In *Pseudomonas aeruginosa*, the *np20* gene, encoding a homologue of the ferric uptake regulator (Fur), was also found to be specifically expressed during infection of mice. Mutational analysis revealed that *np20* is not essential for growth in vitro. However, compared to the wild type, the mutant strain is required in a much higher dose to cause similar lethality in mice (290). Besides regulation of iron uptake, the housekeeping Fur protein is also directly or indirectly involved in the regulation of a substantial number of other genes encoding proteins with remarkably diverse functions, including other regulators, proteins involved in adaptation to oxidative stress, and virulence factors such as exotoxin A (284). Likewise, it was shown that in the plant pathogen *Erwinia chrysanthemi*, besides expression of two high-affinity iron uptake systems, pectate lyase-mediated cell wall degradation is also under control of the Fur regulator (72).

The frequent isolation of genes related to iron homeostasis reflects the importance of iron for microbial growth. Animal pathogens reside in an environment low in iron ions because host proteins such as transferrin and lactoferrin bind iron with high affinity (236). These proteins also play a role in host protection against microbial infection at the mucosal surface by depletion of the available iron (291). Likewise, in certain soils, the plant rhizosphere is scarce in ferrous iron (149, 195). Because iron is essential for microbial growth, the animal pathogens and plant-associated bacteria deploy dedicated systems for high-affinity iron uptake to capture the available iron (287). Furthermore, in several plant and animal pathogens, expression of pathogenesis-related genes is linked to iron availability (72, 206, 228, 283, 284).

Several genes involved in scavenging other metal ions, such as Cu^{2+}, Mn^{2+}, Mg^{2+}, K^{+} and Na^{+} were identified with IVET. Using DFI, the *Streptococcus pneumoniae psaBCA* operon, encoding a manganese uptake system, was identified as being specifically expressed in mice (160). The *psa* promoter is induced more than 10-fold, suggesting an important role during survival within the host. Moreover, *psaB*, *psaC*, and *psaA* mutants are not only growth retarded in medium low in manganese, but are also completely attenuated in infection of mice (161). The *Streptococcus pneumoniae* Psa permease also plays an important role in resistance to hydrogen peroxide and superoxide, in systemic infections, and in nasopharyngeal mouse colonization (168).

In *Sinorhizobium meliloti*, a potassium channel, encoded by *nex10*, is specifically induced in the nodules during alfalfa symbiosis. A *nex10* mutant strain is less efficient in symbiotic nitrogen fixation (188). The *nex10* gene product resembles the regulatory β-subunit of the eukaryotic voltage-gated potassium channels, but the exact function of this type of channel in prokaryotes is unknown. Possible roles in osmoregulation or pH adaptation during symbiosis have been suggested (177, 188).

Amino acid uptake. Although most IVET and DFI studies focused on animal infection systems, genes involved in amino acid acquisition were predominantly isolated from plant-associated bacteria, suggesting that amino acids are available for uptake in the plant environment but much less so in animal hosts. The host-induced expression of genes for amino acid uptake systems was reported for pathogenic bacteria upon plant infection (16, 26) as well as for beneficial bacteria colonizing the plant rhizosphere (218). Part of the amino acids synthesized by plants is exuded into the rhizosphere (113). Although the amount of amino acids in tomato root exudates is not sufficient to sustain rapid growth of plant root-colonizing microorganisms, the amino acids are likely to be taken up and utilized during colonization (249). This is supported by the observation that *Pseudomonas fluorescens* shows chemotaxis towards amino acids present in these root exudates (48).

Acquisition of phosphorus. IVET and DFI studies have revealed host-induced expression of systems for uptake of phosphorus in both animal- and plant-pathogenic bacteria. For instance, the *Shigella flexneri pstS* encodes an ABC transporter for high-affinity phosphate uptake that is specifically expressed during infection of mice. The *Shigella flexneri pstS* mutant strain constructed shows no growth difference in low-phosphate media but causes smaller plaques on macrophage monolayers, suggesting that the loss of *pstS* results in lower infection efficiency (233).

Uptake of sugars and carbohydrates. Several bacterial sugar uptake systems, mostly sugar permeases and sugar-specific phosphotransferase systems (PTS) for monosaccharides (fructose, mannose, and ribose) and disaccharides (sucrose, cellobiose, and maltose), were found to be induced during interaction with several eukaryotic hosts. During passage through the mouse gastrointestinal tract, *Lactobacillus plantarum* seems to

deploy a diverse set of PTS systems for uptake of sugars (24). Sugar uptake systems specifically associated with plant infection were revealed for *Erwinia chrysanthemi* (299). Mutation of *rhiT*, encoding a rhamnogalacturonide transporter, compromises the systemic invasion capability of this phytopathogen (299).

Three genes (*dctS*, *dctD*, and the *dctS* homologue *Rsc1598*) involved in the regulation of C_4-dicarboxylate uptake were isolated with independent IVET screenings for the rhizosphere-colonizing *Pseudomonas fluorescens* (218) and from the phytopathogens *Pseudomonas syringae* (159) and *Ralstonia solanacearum* (26), respectively. C_4-dicarboxylate metabolism is induced in the presence of dicarboxylates and is under the control of regulatory sensor mechanisms. The DctSR two-component regulatory system, which shows high similarity with the FixLJ oxygen sensor system in rhizobia, is necessary for aerobic growth on C_4-dicarboxylates. The DctBD two-component regulatory system is functionally similar to the NtrBC regulatory system that activates expression from σ^{54}-dependent promoters (115). In a similar, ATP-dependent way, DctBD activates expression of *dctA*, encoding a C_4-dicarboxylate:cation (H^+ or Na^+) symporter, which is essential for symbiotic nitrogen fixation (115). C_4-dicarboxylates such as malate and succinate are present in plants and root exudates (9) and are major carbon and energy sources for nitrogen-fixing symbionts (301). IVET studies highlight the significance of this catabolic pathway for other plant-associated bacteria as well.

Miscellaneous nutrients. IVET studies have revealed a variety of other genes specifically expressed in the wild, encoding ABC transporters, porins, and permeases for uptake of diverse components, such as lactate, peptides, choline, and undefined molecules. The potential role of an ABC transporter (ATP-binding subunit RTI006) of *Streptococcus pneumoniae* was revealed by DFI (160). This transporter mediates choline transport and subsequent analysis showed that the *Streptococcus pneumoniae* RTI006 mutant strain shows decreased respiratory tract infection (160). In *Staphylococcus aureus*, choline and its degradation product, glycine betaine, constitute potent osmoprotectants (87, 231). Besides their involvement in uptake of nutrients, substrate-binding components of ABC transporters have also been shown to be implicated in the infection process of some pathogens by facilitating adhesion to host cells, as unequivocally demonstrated in *Streptococcus gordonii* (117) and *Campylobacter jejuni* (205).

Genes Involved in Central Intracellular Metabolism

Intermediary metabolic pathways. As might be anticipated, IVET and DFI screenings revealed the host-induced expression of several genes involved in intermediary metabolic pathways such as the tricarboxylic acid (TCA) and glyoxylate cycles. The glyoxylate pathway enables bacteria to grow on acetate. Interestingly, two genes, *aceA* and *aceB*, of which the corresponding gene products catalyze subsequent steps of the glyoxylate pathway, were isolated with IVET from the animal pathogens *Mycobacterium tuberculosis* (56) and *Yersinia enterocolitica* (300), respectively. An independent SCOTS experiment equally showed that *Mycobacterium tuberculosis* cells contain more *aceA* transcript during macrophage infection (86). Expression of *aceA* and *aceB* may be linked to degradation of host lipids through fatty acid β-oxidation, generating acetyl-coenzyme A for subsequent use as a carbon source (39). Acetyl-coenzyme A entering the TCA or glyoxylate cycle is also generated from pyruvate by the pyruvate dehydrogenase complex. The genes encoding the subunits of this enzyme complex (*aceE* and *pdhC*) have been identified by IVET in *Pseudomonas syringae* upon *Arabidopsis thaliana* infection (16) and by DFI in macrophages infecting *Mycobacterium marinum* (11).

IVET also demonstrated that some TCA cycle genes are upregulated in the host environment. The TCA cycle is a major degradation pathway for generation of ATP but also provides intermediates for biosyntheses. For instance, *fumC*, encoding the fumarase enzyme, showed elevated expression in *Listeria monocytogenes* during infection of mice (77). Analysis of a *Listeria monocytogenes fumC* mutant strain revealed defective growth in phagocytes (77). It is worth noting that *fumC* was also identified in the IVET screening of *Ralstonia solanacearum* upon infection of tomato plants (26).

Reduced coenzymes produced by oxidative metabolism, such as the TCA cycle and fatty acid degradation, can be used to drive ATP synthesis via oxidative phosphorylation. The ATP synthase subunit gene *atpD* was isolated with IVET in a *Staphylococcus aureus* mouse infection model (152). Another gene involved in energy metabolism, *pckA*, was found to be upregulated during *Sinorhizobium meliloti*-alfalfa symbiosis (188). The phosphoenolpyruvate carboxykinase PckA catalyzes the formation of phosphoenolpyruvate from oxaloacetate. It was previously shown that rhizobial *pckA* is strongly induced at the onset of stationary phase or during growth on succinate or arabinose as the sole carbon source, but *pckA* expression is also induced by host root exudates (193). A *Rhizobium pckA* mutant strain revealed a host-dependent symbiotic phenotype, as it lost its ability to establish nitrogen-fixing nodules only in some leguminous plants (194). Using IVET, *pckA* was also found to be induced in *Mycobacterium tuberculosis* during macrophage infection. The host-induced expression of *pckA* was subsequently confirmed with reverse transcription-PCR (56).

Lipid and fatty acid metabolism. IVET, RIVET, and DFI screenings revealed that several microbial genes involved in lipid and fatty acid metabolism are upregulated in animal and plant host environments. Degradation of lipids from host immune cells might protect the pathogen against the host immune response, but, as outlined above, lipid degradation and subsequent utilization of the released fatty acids may also fulfill a nutritional role (32, 157). It was also proposed that pathogen lipases, in combination with fatty acid-modifying enzymes, could inactivate the bactericidal lipids that are produced in host tissue abscesses, thereby increasing survival in this niche (121). This is in agreement with the isolation of the *Staphylococcus aureus lip* gene, encoding a glycerol ester hydrolase, which is specifically induced in host tissue (152).

The upregulation of genes encoding a 3-hydroxyl-coenzyme A dehydrogenase in *Mycobacterium tuberculosis* infecting macrophages was demonstrated independently with IVET (56) and DFI (275). An unexpectedly large number of host-induced genes that are involved in fatty acid metabolism were identified by IVET in *Mycobacterium tuberculosis*, suggesting that fatty acid metabolism is extremely important during life in the host environment. *Mycobacterium tuberculosis* has an astonishingly large number of genes presumed to be involved in β-oxidation

of fatty acids (39). It has been suggested that *Mycobacterium tuberculosis* is able to utilize fatty acids as a major energy source during infection, but it is also possible that fatty acid metabolism is necessary for remodeling the cell envelope upon macrophage infection, thereby evading the host immune response (56). Likewise, Mahan and coworkers found that *fadB*, which is required for β-oxidation of fatty acids, is upregulated in *Salmonella enterica* serovar Typhimurium upon infection of mice and ascribed this to the high concentration of fatty acids encountered by the pathogen during infection (157). FadB homologues were also found to be specifically expressed in *Brucella abortus* and *Mycobacterium tuberculosis* (62, 275).

In the plant pathogen *Erwinia chrysanthemi*, the transcriptional regulator EutR was isolated using IVET (299). This regulator controls the *eut* operon, which is required for ethanolamine utilization (243). Ethanolamine utilization is an important trait for efficient plant infection, since an *Erwinia chrysanthemi eutR* mutant displayed a decreased ability to cause systemic invasion in African violets (299). Ethanolamine, released during degradation of phospholipids, is also a carbon and energy source in the intestinal tract, and the *eut* operon is coregulated with expression of virulence genes (125, 135). In *Salmonella enterica* serovar Typhimurium, the operon involved in ethanolamine utilization is coregulated with genes involved in motility and with genes encoding a TTSS (125).

Carbohydrate metabolism. Several of the genes listed in Table 2 are involved in sugar metabolism. One of these genes, *xylA*, encoding xylose isomerase, was identified in the sugar beet colonizer *Pseudomonas fluorescens* (218). Xylose is a typical plant-derived sugar commonly present in the plant rhizosphere. Xylan is the main carbohydrate found in the hemicellulosic fraction of plant tissues and is hydrolyzed by xylanases into xylose monomers that can be utilized by many bacteria and fungi as a primary carbon source. Xylanase producers are found in all ecological niches where plant material is deposited (210). It is therefore plausible that genes enabling *Pseudomonas fluorescens* to utilize xylose are switched on when the organism is residing in the rhizosphere. It is known that the XylR transcriptional regulator activates *xylA* expression in the presence of xylose (257). The IVET isolation of *xylR* in the plant pathogen *Erwinia chrysanthemi* indicates that xylose metabolism is also activated in planta (299). Notably, *xylA* was also isolated in the mouse gut colonizer *Lactobacillus reuteri* (285). We speculate that *Lactobacillus reuteri xylA* might be induced during survival in the gut by xylose originating from plant material in the mouse feed.

It was mentioned above that promoter traps enabled the isolation of several sugar uptake systems, such as the ribose transport proteins RbsD and RbsC, from *Lactobacillus plantarum* during passage in the gastrointestinal tract of mice (24) and *Haemophilus influenzae* infecting chinchilla, respectively (164). Likewise, other members of the ribose (*rbs*) operon, *rbsR* and *rbsK*, were isolated with IVET from *Lactobacillus plantarum* (24) and from *Klebsiella pneumoniae* (133), respectively, during life in the mouse host. RbsR is a transcriptional repressor of the *rbs* operon, which enables uptake and utilization of ribose. The *rbsR* gene is also upregulated in *Salmonella enterica* serovar Typhi during macrophage infection, as demonstrated with SCOTS (44). An *rbsR* mutant exhibits decreased survival in macrophages compared to the wild type, confirming the importance of *rbsR* induction during macrophage infection (44). Differences in the regulation (repression versus activation) of ribose metabolism in the different host environments might be explained by different sugar contents present in the spleen and intestine or experienced inside macrophages.

Amino acid synthesis. The importance of amino acid uptake for bacterial life in the plant environment was discussed. Likewise, amino acid synthesis seems to be a key trait for interaction and/or survival in the host environment. Members of this class were found to be induced in microorganisms as diverse as the fungal pathogen *Histoplasma capsulatum* and gram-positive and various gram-negative bacteria during interactions with either plant or animal hosts. The overall requirement for amino acids is reflected in the independent isolation of functional homologues of biosynthetic genes, which is the case for *argG*, *hisB*, *ilvA*, *ilvI*, and *lysA* (Table 2).

The importance of amino acid metabolism is illustrated by the mutational analysis of the *Vibrio cholerae argA* gene, initially traced by RIVET, which encodes the *N*-acetyl glutamate synthase involved in de novo synthesis of arginine and proline. Camilli and Mekalanos showed that an *argA* mutant was severely outcompeted by the wild type during infection of mice (32). In an independent RIVET study, the *Vibrio cholerae argH* gene was also shown to be upregulated during infection of mice (191). On the other hand, a *Vibrio cholerae cysI* mutant did not show attenuated pathogenicity, suggesting that arginine biosynthesis plays a more important role in survival of *Vibrio cholerae* during infection of mice than sulfate assimilation for cysteine biosynthesis (32). Another promoter identified with IVET drives expression of the *Salmonella enterica* serovar Typhimurium *carAB* operon, which encodes carbamoylphosphate synthetase, involved in arginine and pyrimidine synthesis (156). Construction of a *carAB* mutant strain revealed that expression of this operon is critical for full virulence (156). SCOTS analysis of macrophage-infecting *Mycobacterium avium* also revealed *carA* expression (105). However, a putative antisense α-*carA* transcript was identified with IVET in *Porphyromonas gingivalis* (298).

Amino acid catabolism. In contrast to the genes involved in amino acid synthesis that were isolated ubiquitously, only a few genes involved in amino acid degradation were found to be induced in the wild. Three genes (*gcvP*, *gcvH1*, and *gcsH1*) involved in glycine cleavage were specifically induced during plant infection by *Ralstonia solanacearum* (26) and *Pseudomonas syringae* (16) and during gastrointestinal tract transit of *Lactobacillus plantarum* (24), respectively.

Pseudomonas bacteria colonizing the rhizosphere of plants apparently also adapt their metabolism by activating specific catabolic pathways, as illustrated by the IVET identification of genes probably involved in the degradation of plant-derived compounds (78, 218, 224). In *Ralstonia solanacearum*, two genes involved in the metabolism of glycolate were found to be upregulated during tomato infection (26). Glycolate is produced during photorespiration (28), and it appears that this phytopathogen activates a catabolic pathway for using this plant-derived compound as a carbon source.

Nucleotide synthesis. In several IVET studies with auxotrophy-based selection, knockouts in nucleotide biosynthesis genes (*purA*, *purEK*, *pyrB*, *ura5*, and *thyA*) were used as genetic

background because the corresponding mutants were demonstrated to be severely attenuated in growth in the host environment (Table 1) (92, 93, 98, 140, 156, 226, 264, 290). It is therefore not surprising that application of promoter traps showed elevated expression of several genes involved in nucleotide metabolism in plant- or animal-colonizing or -infecting bacteria. These include genes involved in de novo nucleotide biosynthesis as well as genes involved in salvage pathways for pyrimidine or purine nucleotides.

Protein synthesis and degradation. In class III, a subgroup of genes involved in protein synthesis can be distinguished. The repeated isolation of several genes encoding amino acid tRNA synthetases together with the gene for ribosomal recycling factor (Rrf) drew attention to the importance of the translation process. For instance, using DFI, the arginyl tRNA synthetase encoded by *argS* was shown to be upregulated in *Mycobacterium marinum* during macrophage infection (11). The same gene was isolated in *Mycobacterium avium* in a SCOTS screen (105).

In diverse microorganisms, genes involved in protein folding were identified. A thiol-disulfide interchange protein, encoded by *dsbD*, was isolated with IVET from *Pasteurella multocida* (108). DsbD is a membrane-bound enzyme that is responsible for maintaining DsbC in the reduced state by translocating electrons from thioredoxins in the cytoplasm. DsbC is a periplasmic disulfide bond isomerase/reductase that catalyzes the rearrangement of disulfide bonds. In this way, improper protein folding resulting from inaccurate disulfide bonds is corrected (119). The *dsbD* gene was also isolated by STM screening, demonstrating that a *Pasteurella multocida dsbD* mutant strain is attenuated in virulence (75). Interestingly, the *dsbB* gene of *Haemophilus influenzae* was also isolated by means of IVET (164). The membrane-bound DsbB transfers electrons to the respiratory chain to keep DsbA in the oxidized form. DsbA is a strong thiol oxidant that catalyzes disulfide bond formation of proteins that are exported to the periplasm (119). As proper folding, stability, or activity of extracellular proteins often relies on the formation of disulfide bonds, *dsbB* and *dsbD* may be involved in maturation and stability of virulence factors or secreted toxins and thereby play a role in pathogenesis. It is therefore not surprising that *dsbA* mutants of various pathogens display avirulent phenotypes (119, 204).

Conversely, IVET and DFI enabled the isolation of several genes involved in protein degradation. In *Yersinia enterocolitica*, a protease encoded by *hreP* was found to be upregulated during infection of mice. Mutational analysis revealed a strong reduction of competitiveness and virulence of the *hreP* mutant (300). The HreP protease shows significant similarity with eukaryotic subtilisin/kexin-like proprotein convertases and was probably acquired by horizontal gene transfer (100). Although mutational studies unequivocally demonstrated the importance of HreP in virulence, its precise role has yet to be elucidated. In several bacterial pathogens, proteases were identified as virulence factors (274).

Cofactor biosynthesis. Another subgroup comprises the genes involved in the biosynthesis of several cofactors, such as biotin, ubiquinone, nucleotide cofactors, thiamine, and iron-containing cofactors such as heme and Fe-S clusters. For instance, three host-induced genes that are involved in heme biosynthesis (*hemA*, *hemB*, and *hemD*) were isolated with IVET from *Salmonella enterica* serovar Typhimurium (98), *Ralstonia solanacearum* (26), and *Yersinia enterocolitica* (300), respectively. It has been postulated that heme synthesis may add to protection against oxidative stress by serving as a catalase cofactor (98). The IVET-identified *Sinorhizobium meliloti nifS* gene encodes a cysteine desulfurase known to be required for nodulation (188). NifS supplies the inorganic sulfide for the formation of the Fe-S clusters in the nitrogenase enzyme, which catalyzes nitrogen fixation during symbiosis. Expression of the nitrogenase enzyme is tightly regulated and known to be specifically induced during symbiosis.

Genes Involved in Adaptation to Environmental Stresses

Oxidative stress. IVET studies exploring bacterial life in various host environments have revealed a variety of mechanisms to cope with oxidative stress. Life in the presence of oxygen poses a permanent threat due to the generation of reactive oxygen species in biological systems (110). Furthermore, plants and animals harnessed this toxic potential as a biological weapon against invading microorganisms. During host infection, animal pathogens are frequently exposed to reactive oxygen species, such as superoxides, hydrogen peroxides, or organic peroxides, as a result of the release of lysosomal contents within inflammatory cells (29). It is also known that plant cells induce a series of defense responses against pathogens, including the generation of reactive oxygen species such as superoxide and hydrogen peroxide, also known as the oxidative burst (134).

Several IVET screens revealed genes playing a role in glutathione synthesis or glutathione reduction that are upregulated during interaction with plant or animal hosts. Glutathione is an important biomarker for oxidative stress. It plays a major role in protection against oxidative stress by dismantling free radicals but is also important for protection against other stresses, such as detoxification of hazardous chemicals or heavy metals (97, 107, 162).

Thiol-specific antioxidants comprise a broad class of antioxidant enzymes that are involved in protection against oxidative stress, and several members of this class were identified with promoter traps. Peroxiredoxins are widespread in the *Archaea*, *Bacteria*, and *Eukarya* and function as antioxidants by reducing peroxides, thereby preventing damage to biomolecules (297). The bacterioferritin comigratory protein encoded by *bcp* is a peroxiredoxin (118) that was found to be upregulated in both *Ralstonia solanacearum* during tomato infection (26) and *Pseudomonas stutzeri* during rice colonization (224). In *Sinorhizobium meliloti*, a peroxiredoxin, encoded by *nex1*, was shown to be involved in the symbiotic interaction with alfalfa, since nitrogen fixation is slightly impaired in a *nex1* mutant strain. Using the *gusA* reporter contained in the promoter trap, Oke and Long (188) also showed that *nex1* is expressed in a restricted zone of the nodule, more specifically in the nodule tip. This constituted the first evidence of bacterial antioxidant activity in nodules. Recently it was also shown that in *Rhizobium etli*, the peroxiredoxin PrxS is expressed during the symbiotic interaction with its host, thereby protecting *Rhizobium etli* cells in the nodules (51). The frequent IVET isolation of genes required for inactivation of reactive oxygen species indicates that for bacteria interacting with plants, oxidative dam-

age of biomolecules represents a major form of stress. It can be seen from Table 2 that plant colonizers frequently express peroxidases or catalases for direct inactivation of peroxides, whereas only one example was found in an animal pathogen. The *Actinobacillus pleuropneumoniae ohr* gene, encoding an organic hydroperoxide reductase, is induced during pig infection. It was shown that *ohr* expression could be induced by organic peroxides such as cumene hydroperoxide but not by paraquat or hydrogen peroxide (240).

Peptide methionine sulfoxide reductases, encoded by *msrA* and *msrB*, are important antioxidant enzymes that mediate the repair of proteins damaged by sulfoxidation of methionine residues (1, 292). The genes are upregulated in both spinach-infecting *Erwinia chrysanthemi* (299) and mouse-infecting *Lactobacillus reuteri* (285).

Acid stress. Gastrointestinal pathogens have to cope with acid stress during transit of the gastric acid barrier before colonizing the intestine. Polyamines, such as cadaverine and spermidine, play a role in the physiological adaptation process known as the acid tolerance response (71, 200). The transcriptional regulator *cadC* was isolated with IVET from *Salmonella enterica* serovar Typhimurium following intragastric infection (98). CadC controls expression of the cadaverine-generating lysine decarboxylase CadA, which is an important component of the acid tolerance response (200). Decarboxylation of lysine produces cadaverine and carbon dioxide, thereby consuming a cytoplasmic proton. Cadaverine is subsequently excreted and exchanged with lysine by the *cadB*-encoded lysine/cadaverine antiporter. The CadC-mediated upregulation of *Salmonella enterica* serovar Typhimurium *cadA* is in line with results from SCOTS which also showed that *cadA* is specifically expressed during macrophage infection by *Salmonella enterica* serovar Typhi. Analysis of a *Salmonella enterica* serovar Typhi *cadA* mutant exhibited decreased survival in macrophages, especially in the early stage of infection (44).

In addition, RIVET enabled the identification of *Vibrio cholerae cadA* as a gene induced during infection of mice (174). *Vibrio cholerae* is only weakly resistant to acid stress, and it was originally thought not to possess an acid tolerance response. However, using IVET, it was shown for the first time that *cadA* is upregulated in *Vibrio cholerae* during infection of mice. CadA was subsequently demonstrated to play an important role in the acid tolerance response in *Vibrio cholerae*. However, mutational analysis revealed that *cadA* is not essential for full virulence (174), probably because other amino acid decarboxylases are still functional and because amino acid decarboxylases represent only part of the overall acid tolerance response (70).

The *speF* gene encodes ornithine decarboxylase, which catalyzes synthesis of putrescine, thereby consuming a cytoplasmic proton. Expression of this gene in *Pasteurella multocida* during infection of mice was detected with IVET (108). Likewise, the *spe2* gene was captured with IVET from mouse-infecting *Haemophilus influenzae* (226). This gene encodes a putative *S*-adenosylmethionine decarboxylase and is probably involved in increasing polyamine synthesis during infection.

Some polyamines, such as putrescine, are found in plant root exudates. During plant root colonization, *Pseudomonas fluorescens* upregulates a gene involved in putrescine uptake (*potF2*) (218). This observation is consistent with the ability of *Pseudomonas fluorescens* to utilize putrescine as sole nitrogen source (132). Degradation of putrescine during infection of tomato plants by *Ralstonia solanacearum* is suggested by the upregulation of a *gabD1* homologue, encoding a putative succinate semialdehyde dehydrogenase (26). This phytopathogen may use this plant-derived compound as a carbon and nitrogen source, but at the same time, interference with the plant's defense system may be exerted. Putrescine is a precursor in the synthesis of spermine that has been implicated in the induction of the hypersensitive response (286).

Osmotic stress. The *Yersinia enterocolitica mdoH* gene was found to be specifically induced during infection of mice (300). MdoH is involved in the synthesis of periplasmic β-glucans and might protect *Yersinia enterocolitica* against osmotic stress. It is known that periplasmic β-glucans, which form intrinsic components of the gram-negative bacterial envelope, improve survival in low-osmolarity environments (179). Periplasmic β-glucans are also required for full virulence of the plant pathogens *Erwinia chrysanthemi* (198) and *Pseudomonas syringae* (150).

A *Ralstonia solanacearum mdoG* homologue encoding a putative glucosyl transferase for β-1,2-glucan synthesis was identified during an IVET study of tomato infection (26). In *Rhizobium leguminosarum* it was demonstrated that the *ndvB* gene, which is involved in the synthesis of cyclic β-glucans, is specifically expressed during symbiosis (3). These low-molecular-weight cell surface carbohydrates are known to be required for root infection and are thought to function as suppressors of a host defense response. *ndvB* homologues are found almost exclusively in members of the *Rhizobiaceae*, such as *Sinorhizobium meliloti*, *Agrobacterium tumefaciens*, and *Brucella abortus*, and transcription of the *ndvB* gene is usually repressed in conditions of high osmolarity (21, 46). An *ndvB* homologue was recently identified in *Pseudomonas aeruginosa*, and it was demonstrated that a *Pseudomonas aeruginosa ndvB* mutant was not able to develop high-level biofilm-specific antibiotic resistance. Mah and coworkers (153) discovered that periplasmic glucans can interact with antibiotics and suggested that the antibiotics may thereby be arrested in the periplasm, preventing them from reaching their sites of action.

Detoxification by efflux systems. Several host-induced genes are involved in divalent metal transport. Cation efflux pumps putatively involved in detoxification of cations and heavy metals were isolated independently with IVET in several bacteria during interaction with plant and animal hosts. For instance, a copper-transporting ATPase (CopA) is specifically expressed in *Lactobacillus plantarum* during colonization of the gastrointestinal tract (24) and *Staphylococcus aureus* during infection of mice (152). Although the exact biological role during interactions with plant or animal hosts is not well known, bacterial cation efflux pumps are widespread and allow detoxification when the heavy metal concentration in the cells reaches toxic levels (248).

Using IVET, homologues of genes encoding components of a resistance-nodulation-cell division (RND)-type multidrug efflux pump, *acrF* and *acrA*, were isolated from plant-associated *Pseudomonas fluorescens* (78) and *Ralstonia solanacearum* (26), respectively. In *Escherichia coli*, the *acr* genes are involved in acriflavine resistance. In *Yersinia enterocolitica*, the transcriptional regulator (*acrR*) of the acriflavine efflux pump was also

shown to be expressed during infection of mice (300). In addition, drug efflux pumps and other proteins involved in antibiotic resistance were shown to be up-regulated in the host environment.

Finally, quite a few genes, encoding heat shock proteins and proteins involved in general stress response, were isolated as well.

Regulatory Genes

The promoter trapping of numerous regulatory genes reflects the ability to respond to environmental challenges through changes in the gene expression profile during interaction with a host. In Table 2, regulatory systems that control specific and well-defined processes are listed together with their target genes, and some are discussed in the corresponding sections. This class comprises genes encoding global regulators, regulatory genes that control expression of genes with unknown function or have unknown target genes.

Several of the IVET-identified regulatory genes encode two-component regulatory systems. These regulatory systems permit organisms to respond to changes in their environment and are often associated with global regulatory systems as well as with regulation of virulence. Sensor kinases are able to perceive external stimuli and can activate the response regulator that subsequently acts as a transcriptional regulator (reviewed in reference 293). However, response regulators are not uniquely activated by the corresponding sensor kinase. For instance, it has been shown that several response regulator proteins can be phosphorylated by acetyl phosphate, a significant secondary source of phosphoryl groups (128, 171, 266). Therefore, the two key enzymes of acetyl phosphate metabolism, acetate kinase (encoded by *ack*) and phosphotransacetylase (encoded by *pta*), can indirectly modulate some regulatory pathways. IVET isolation suggests a possible role for *pta*-encoded phosphotransacetylase during rice root colonization by *Pseudomonas stutzeri* (224). Also, in *Pasteurella multocida*, an *ack-pta* operon was shown to be specifically expressed during infection of mice (108). Interestingly, the *Vibrio cholerae pta* gene was previously isolated using the STM screening for genes required for colonization of the host intestine, implying that a *Vibrio cholerae pta* mutant strain is significantly reduced in virulence efficiency (36). Acetyl phosphate can also serve as an energy source. In *Bradyrhizobium japonicum* bacteroids (208) and in *Azotobacter vinelandii* (22), acetyl phosphate is used as an energy source to support nitrogen fixation.

Vibrio cholerae vieB is a well-characterized example of an IVET-isolated two-component response regulator (32). Since no tested signals have been able to induce *vieB* expression in vitro, traditional in vitro studies would probably not have revealed this gene, underscoring the potential of IVET as a tool to explore gene expression in complex conditions that are not easy to mimic in vitro. *vieB* is part of the *vieSAB* cluster with *vieS*, encoding the constitutively expressed sensor kinase, and *vieA* and *vieB*, encoding two distinct response regulators (136, 175). Although Camilli et al. (32) found that a *Vibrio cholerae vieB* mutant exhibited small but reproducible colonization defects, Lee et al. (136) demonstrated that the wild-type strain had no competitive advantage during colonization.

VieB is an atypical response regulator, because, in contrast to VieA, it lacks the C–terminal DNA binding domain. It is therefore hypothesized that VieB might modulate the phosphorylation state of VieA by competing for phosphate of phosphorylated VieS. RIVET was used to demonstrate that *vieB* is only expressed during the infection and enabled analysis of spatial and temporal *vieB* expression patterns for further elucidation of the possible role of *vieB* during infection (136). In addition, it was shown with a modified RIVET technique, designed to isolate regulators of *ctxA* (encoding a subunit of the cholera toxin) or *toxT* (encoding an important virulence regulator), that the *vieSAB* regulatory system is required for full expression of cholera toxin during infection as well as in in vitro conditions (137, 139, 272).

The *Yersinia enterocolitica yeiE* gene, encoding a LysR-like transcriptional regulator, was also found to be up-regulated during infection of mice, suggesting a possible role in virulence by either positive or negative gene regulation (300). Knocking out *yeiE* apparently resulted in a more aggressive pathogen in the initial stage of infection. However, the *Vibrio cholerae yeiE* mutant strain caused less mortality than the wild type in later stages of infection, suggesting that fast spread and aggressive infection are not necessarily linked to high virulence (300).

Some global regulators, such as *himA* and *hfq*, are found to be upregulated in the host environment. The *Salmonella enterica* serovar Typhimurium *himA* gene encodes the α-subunit of the integration host factor and was identified as a host-induced gene with both IVET (156) and DFI (279). Application of DFI revealed a 15-fold induction of *himA* during macrophage infection (279). The integration host factor is an auxiliary DNA-binding protein that is involved in gene regulation, DNA replication, and recombination (74). In addition, it was observed with DFI that the *Haemophilus influenzae ihfB* gene, encoding the β-subunit of the integration host factor, was induced during chinchilla infection. Subsequent reverse transcription-PCR analysis confirmed a 2.5-fold upregulation (164).

The *hfq* gene encodes the so-called host factor I (HF-I, Hfq) and was shown to be specifically induced in *Pseudomonas stutzeri* during rice colonization (224). Moreover, the equivalent DNA region in *Yersinia enterocolitica*, containing the open reading frames *mutL*, *miaA*, *hfq*, and *hflX*, was also isolated with IVET (300). Hfq probably has a general regulatory role, because it is necessary for efficient translation of the alternative sigma factor RpoS, which in turn controls the expression of several genes in the stationary phase. But Hfq is also involved in regulation by affecting the stability of mRNAs that are involved in DNA damage repair (*mutS*) and modification of the outer membrane (*ompA*) (253). Furthermore, Hfq plays an important role in the interaction of certain pathogenic bacteria with their host. The Hfq homologue of *Yersinia enterocolitica* positively regulates expression of an enterotoxin gene (*yst*), involved in the expression regulation of virulence factors (186). It was also shown that an *hfq* homologue (*brg*) in the phytopathogen *Erwinia carotovora* is necessary for synthesis of low-molecular-weight bacteriocins (38), again suggesting an important role of *hfq* in bacterial survival and enhanced competitiveness in the rhizosphere. Hfq is also essential for the persistence of spleen infection by species of the animal pathogen *Brucella*. *Brucella* mutants that lack the *hfq* gene are more sensitive to acidic conditions and oxidative stress (230).

Besides transcriptional gene regulation, some host-induced genes are involved in posttranslational regulation. IVET enabled the identification of serine/threonine protein kinases that are specifically expressed in *Pseudomonas aeruginosa* (290), *Lactobacillus plantarum* (24), and *Streptococcus gordonii* (127) during interaction with mouse and rabbit hosts. Serine/threonine kinases are ubiquitous in both prokaryotic and eukaryotic cells and are thought to play a central role in signal transduction. Moreover, some serine/threonine kinases are known to be indispensable for virulence (79). The *Pseudomonas aeruginosa* serine/threonine protein kinase encoded by the IVET-traced *ppkA* gene was subjected to mutational analysis. Compared to the wild type, a *ppkA* null mutant revealed similar growth in vitro, but a clear difference in virulence was observed. A time delay in animal death was observed when mice were infected with the *ppkA* mutant and a 10-fold-higher initial inoculum was required to cause similar disease effects in mice compared to wild-type *Pseudomonas aeruginosa* (288). However, since the nature of the substrates of PpkA kinase activity is unknown, no exact function for *ppkA* in virulence regulation can yet be assigned (185).

Genes Involved in Cell Envelope Structure and Modification

Peptidoglycan layer. Genes involved in modification or recycling of the peptidoglycan structure have been isolated repeatedly with IVET or DFI (Table 2), not only from several mammalian pathogens but also from plant-pathogenic bacteria. One such example is *Pseudomonas syringae ampG*, which is involved in peptidoglycan recycling and is induced during *Arabidopsis thaliana* infection (16). This is in agreement with the finding in *Ralstonia solanacearum* that a knockout of *ampD*, involved in peptidoglycan synthesis, results in decreased plant infection (271). STM showed that *Neisseria meningitidis* AmpD is necessary during rat infection (267).

Another gene involved in peptidoglycan synthesis encodes the *N*-acetylmuramoyl-L-alanine amidase and was isolated with IVET and RIVET from *Mycobacterium tuberculosis* (56) and *Staphylococcus aureus* (152). These results indicate that the bacterial cell envelope is an important determinant for the establishment of a bacterium-host interaction. In line with this, it was observed that *Salmonella enterica* serovar Typhimurium peptidoglycan undergoes structural alterations that probably add to its fitness when it resides in mammalian cells (17, 216).

Another enzyme specifically expressed during plant and animal pathogenesis is the lytic transglycosylase, of which homologues were isolated with IVET or DFI in *Shigella flexneri* (14), *Pseudomonas syringae* (16), *Brucella abortus* (62), and *Erwinia chrysanthemi* (299). The lytic transglycosylase, involved in recycling and remodeling of the peptidoglycan (14), is thought to be required for the integration of various macromolecular transport systems in the cell wall (130). It was shown that the lytic transglycosylase encoded by the *Vibrio cholerae sltA* gene is in some way involved in the regulation of *toxT* in *Vibrio cholerae*. ToxT is a transcriptional regulator belonging to the virulence gene regulatory cascade. It was subsequently shown that a mutation in *sltA* affects colonization fitness (137). This is in line with the observation in *Shigella flexneri* that a lytic transglycosylase encoded by *sltY* is specifically expressed during infection of HeLa cells but not during survival in macrophage cells. The subsequently constructed *Shigella flexneri sltY* mutant exhibited attenuated virulence (14).

In contrast to the *Shigella flexneri* transglycosylase, the peptidoglycan transglycosylase MtgA in *Brucella abortus* is upregulated during macrophage infection (62). By mutational analysis, an IVET-identified lytic transglycosylase gene, *ipx10*, encoding a homologue of *Escherichia coli* MetD, was also shown to be implicated in the virulence of a plant pathogen, *Pseudomonas syringae* pv. *tomato*, on *Arabidopsis thaliana* (16). The host-induced *Pseudomonas syringae ipx10* gene is located in the so-called conserved effector locus. It is thought to play a role in facilitating the assembly of the TTSS into the peptidoglycan. Expression analysis showed that *ipx10* exhibited sixfold induction during infection. Decreased virulence was observed in a *Pseudomonas syringae ipx10* mutant strain (16). Notably, the *ipx10*-encoded transglycosylase shares the soluble lytic transglycosylase (SLT) domain with the SltY transglycosylase, which was also shown to be required for *Shigella flexneri* virulence (14), as mentioned above.

Surface-exposed components. Several genes involved in lipopolysaccharide biosynthesis were found to be induced in animal pathogens during survival within the host. Lipopolysaccharides are well-defined virulence factors, and the structure and decoration of lipopolysaccharides are strictly regulated (144). Expression of genes involved in the biosynthesis of lipid A, a major constituent of lipopolysaccharides, is induced by the transcriptional regulator PhoP (223). Interestingly, the *Salmonella enterica* serovar Typhimurium *phoP* gene was found to be upregulated during mouse infection. The activity of the PmrA-PmrB two-component system, that is equally upregulated during infection (98), is modulated by the PhoP-PhoQ two-component system (122). Activation of PmrA-PmrB leads to modification of lipopolysaccharide and thereby confers resistance to cationic antibiotic polypeptides, which may allow bacteria to survive within macrophages (90).

In several bacteria, adhesion molecules are induced during interaction with the host. For example, in *Porphyromonas gingivalis*, the *hagB* and *hagC* genes, both encoding hemagglutinins, were identified with IVET (141, 298). Furthermore, in *Sinorhizobium meliloti*, the putative adhesion molecule encoded by the host-induced *nex18* gene proved important for symbiosis, as disruption of *nex18* resulted in reduced nitrogen fixation (188). Nex18 is a fasciclin I-like protein containing a predicted signal peptide, suggesting that *nex18* is secreted. Fasciclin I is an adhesion molecule found in some eukaryotes (58).

Outer membrane proteins. Several IVET and DFI studies demonstrated the importance of lipoproteins and other outer membrane proteins during life in the host environment (Table 2). For instance, IVET demonstrated the upregulation of two genes (*pcp* and *glpQ*), encoding membrane-associated lipoproteins, in *Pasteurella multocida* during infection of mice (108). Although no enzymatic function could be assigned to Pcp, it was demonstrated that Pcp is surface exposed (148). Surface-exposed proteins probably contribute to the modulation of bacterial cell surface properties during interaction with the host, thus playing a role in evading the host immune response. As GlpQ is not surface exposed, it is probably not involved in host immunity but might still play a role in pathogenesis. Based

on its glycerophosphodiester phosphodiesterase activity, GlpQ is predicted to play a role in the utilization of deacylated phospholipids originating from mucosal secretions, thereby enabling the bacteria to multiply in the mucus layer (148). Another outer membrane protein, encoded by *sif15*, displays higher expression in *Yersinia enterocolitica* during systemic infection of mice (83). The *sif15* mutant is drastically attenuated in virulence compared to the wild type after intraperitoneal coinoculation (83).

One of the isolated host-induced porin genes, the *Vibrio cholerae*-encoded *vca1008* gene (32), was studied in more detail to assess its role in mouse virulence. The close relationship with another *Vibrio cholerae* porin, OmpU, suggests that OmpU and Vca1008 are paralogues and might have overlapping functions. This can explain why an *ompU* mutant strain does not show defective growth or virulence (214). Likewise, a *Vibrio cholerae vca1008* mutant shows no defective growth in vitro. However, during infection of mice, the *vca1008* mutant strain is severely outcompeted by the wild-type strain, indicating that the Vca1008 porin is necessary and sufficient for virulence, while OmpU is dispensable (192).

IVET enabled the identification of the *Pseudomonas fluorescens wssE* gene as being specifically induced in sugar beet rhizosphere (78). *wssE* encodes a cellulose synthase subunit and is part of the *wss* operon for synthesis of acetylated cellulose polymers (259, 260). It is thought that this polymer functions in colony development and bacterial cell-cell contact rather than mediating adhesion to the plant surface (78, 259). It was also demonstrated that the *wss* operon contributes to ecological fitness on the leaf surface and, to a minor extent, to ecological fitness in the sugar beet rhizosphere (78).

The cell envelope is crucial for communication and interaction with host cells. Surface-exposed proteins, such as adhesins, can mediate cell-cell contact by anchoring the bacterium to the surface of the host tissue. Alterations of the cell wall during the interaction might be critical for adaptation to the different stresses encountered during life in the host environment. In addition, by changing the cell surface topology, pathogens can escape the host immune response.

The frequent isolation of cell envelope genes by IVET and DFI reflects the important role played by the bacterial cell surface in the interaction with other microorganisms. This is in agreement with microarray data showing that approximately 70% of the *Salmonella enterica* serovar Typhimurium genes that are involved in cell surface structure and over 20% of the genes involved in cell envelope biogenesis and outer membrane proteins are differentially expressed during macrophage infection (60).

Genes Involved in Virulence and Secretion

Several genes encoding components of different types of secretion machineries were isolated with promoter traps. In *Erwinia chrysanthemi*, genes involved in the type II secretion pathway, *outF* and *outG*, are induced during spinach infection (299). Pectinases, which are major virulence factors in the soft rot-causing *Erwinia chrysanthemi*, are secreted through the Out system (123, 273). It was also demonstrated with IVET that genes involved in pectin degradation, *plyD* and *pme*, are upregulated during plant infection by the plant pathogens *Pseudomonas syringae* (16) and *Ralstonia solanacearum* (26), respectively. In tomato-infecting *Ralstonia solanacearum*, a gene encoding GspK, a component of the type II secretion system, is also induced. This secretion system is required for full virulence of this phytopathogen (120).

Type III secretion system. The majority of genes involved in secretion, identified through IVET and DFI in several animal and plant pathogens, encode components of a TTSS. Many bacterial pathogens of plants and animals use this specialized machinery to deliver virulence effector proteins across the bacterial cell envelope into host cells (reviewed in references 2, 31, 40, and 106). Besides translocating effector molecules, it was demonstrated in *Escherichia coli* that TTSS also mediates the secretion of adhesion molecules such as intimin (187).

In *Salmonella enterica* serovar Typhimurium, DFI enabled the isolation of a macrophage-inducible TTSS component encoded by *ssaH* (279). *ssaH* exhibited a 400-fold induction during growth in macrophages compared to in vitro growth. An *ssaH* mutant strain was severely outcompeted by the wild type in a mouse infection model, pointing to the importance of a functional TTSS during macrophage infection (99, 279). Interestingly, application of DFI also revealed the host-induced expression of an effector protein encoded by *sifA* in *Salmonella enterica* serovar Typhimurium, suggesting a coordinated expression pattern of the secreted protein and its secretion system (30). *sifA* appeared to be essential for *Salmonella enterica* serovar Typhimurium virulence as well, since an *sifA* mutant was severely outcompeted by the wild type in a mouse infection model (15).

DFI analysis of *Salmonella enterica* serovar Typhimurium infection revealed that another TTSS effector (PipB) is only expressed during mouse infection (30). *pipB* is part of a *Salmonella* pathogenicity island and is thought to be involved in glycolipid biosynthesis (296). It was demonstrated that PipB associates with host intracellular membranes, thereby possibly allowing specific interactions with host membrane molecules (129). This DFI application confirmed an earlier observation of Pfeifer et al. (209), who demonstrated, by random insertion of a *lux* reporter gene into the genome, that *pipB* is upregulated during mouse infection. The *pipB* mutant strain that resulted from the *lux* insertion is slightly attenuated in infection of mice, demonstrating a specific virulence function of the host-induced *pipB* gene (209).

One of the *Pseudomonas aeruginosa* open reading frames identified through IVET using the burned-mouse infection model (92), PA2808, was shown to encode a novel TTSS-regulatory protein, PtrA, inhibiting the activity of the transcriptional activator ExsA through a direct interaction (91). Although highly conserved among *Pseudomonas aeruginosa* strains, no *ptrA* homologues have been found in other bacteria, including other TTSS-containing *Pseudomonas* species.

In the plant pathogens *Pseudomonas syringae* (16) and *Erwinia chrysanthemi* (299), several components of the TTSS along with effector proteins were shown to be upregulated during plant infection. The *Erwinia chrysanthemi hrpB* gene plays a vital role in plant infection, since a mutation in *hrpB* severely reduces plant pathogenesis. Reduced virulence was also observed when two other *hrpB*-linked genes, *hrcJ* and *hrpD*, were knocked out. Notably, in the same cluster, a gene encoding a lytic murein transglycosylase was found, but unlike

Pseudomonas syringae ipx10, the *pup2D* gene is not essential for virulence (299). DFI trapping of HrpL-regulated promoters has been implemented in genomewide screens for TTSS effector proteins in two *Pseudomonas syringae* pathovars (35).

Unexpectedly, an *hrcC* homologue, *rscC*, was also isolated with IVET from the nonpathogenic *Pseudomonas fluorescens* SBW25 (218). The *rscC* gene is specifically expressed in the sugar beet rhizosphere, but in contrast to *Pseudomonas syringae hrcC*, it was not induced upon leaf colonization. Although a *Pseudomonas fluorescens hrcC* mutant is not attenuated in sugar beet root colonization, the gene cluster encoding the TTSS in *Pseudomonas fluorescens* is functional. The biological significance of TTSS upregulation in the rhizosphere in this particular case remains to be determined (212). Among plant-associated bacteria, TTSSs were previously only reported in pathogenic bacteria and symbiotic rhizobia. However, DNA hybridization and PCR amplification experiments indicate that TTSSs are more widespread than originally thought (167, 212, 215, 227).

Virulence factors. Another subgroup of class VII includes major virulence factors, such as the *Salmonella enterica* serovar Typhimurium *spvB* gene (98). SpvB is predicted to be a secreted protein containing a carboxy-terminal ADP-ribosyltransferase domain (84, 196). ADP-ribosylation activity is a well-known feature of certain bacterial toxins by which target proteins in their animal hosts can be inactivated. The virulence function of the clustered *spvABCD* genes is not known, but these genes were shown to be involved in systemic infection by increasing the replication rate of the bacteria in host tissues beyond the intestines (88, 89). Although an *spvB* mutation does not result in a significant virulence defect in the early stages of infection (98), it was shown that *spv* genes are essential for systemic infection at a later time of infection. A clear decrease in intracellular proliferation and in epithelial cell apoptosis was observed during infection by the *spv* mutants (15, 197).

In addition, DFI identified the *mig5* gene, encoding a putative lipoprotein, which is located on the *Salmonella enterica* serovar Typhimurium virulence plasmid, approximately 2 kb upstream of the *spv* virulence operon. *mig5* exhibits 24-fold induction during growth in macrophages, and a *Salmonella enterica* serovar Typhimurium *mig5* mutant shows decreased infection ability (279). Another DFI study revealed granuloma-specific expression of genes encoding members of the proline glutamic acid-polymorphic GC-rich repetitive sequence (PE-PGRS) family in *Mycobacterium marinum* infecting frogs (221). Mutations in these genes prevented replication in macrophages and impaired persistence in granulomas.

The *Porphyromonas gingivalis ivi11* gene, encoding an immunoreactive antigen, is another example of a novel IVET-isolated gene that was subsequently shown by mutational analysis to contribute to infection efficiency and survival in the host. IVET confirmed its significance during *Porphyromonas gingivalis* infection of mice (298).

The hemolysin homologues *ispA* and *yhdP* were also shown to be specifically expressed in *Lactobacillus plantarum* and *Listeria monocytogenes*, respectively, during their interaction with murine hosts (24, 295). A *Listeria monocytogenes yhdP* mutant exhibited decreased survival in murine spleens, indicating an important role for the *yhdP*-encoded hemolysin homologue in the infection process (295). In *Yersinia ruckeri*, the hemolysin activator protein (ShlB) is also specifically expressed during fish pathogenesis (65). Notably, listeriolysin-encoding *hlyC* was used as a selection tool in the *Listeria monocytogenes* IVET screen (77) (Table 1). Expression of hemolysins causes lysis of red blood cells. The released hemoglobins can be dissociated into heme, which can subsequently be utilized as an iron source by many pathogenic bacteria (232). Therefore, secreted hemolysins represent important virulence factors in several animal pathogens, but they also provide a target for vaccine development (50).

Using RIVET, expression of *ctxA*, encoding a subunit of cholera toxin during *Vibrio cholerae* infection of mice, was confirmed (32). This represents an example of a prophage-encoded virulence factor. Upregulation of several phage-derived genes in *Escherichia coli* during chicken infection was also demonstrated using SCOTS (54). This phenomenon is not restricted to animal pathogens, as expression of several phage-derived genes was also detected with the *Ralstonia solanacearum*/tomato IVET system (26). It has been proposed that phage-encoded factors can contribute to the fitness of their lysogenic host in different ecological niches (27).

Genes Involved in Nucleic Acid Metabolism

This class of host-induced genes contains several DNA synthesis and modification genes from various microorganisms. For instance, the *Streptococcus pneumoniae* DNA topoisomerase IV, encoded on the DFI-identified transcriptional fusion RTI004, is upregulated during macrophage infection (160). Topoisomerases are required during every step in the replication process. Topoisomerase, together with gyrase, influences the superhelicity of DNA (145). Other genes of this class might be involved in DNA supercoiling, which has been reported to regulate virulence gene expression in *Shigella flexneri* (52). Helicases, also involved in modifying DNA topology, were upregulated in *Yersinia enterocolitica* infecting mice (83) and in *Pseudomonas fluorescens* colonizing sugar beets (302). It was shown that a *Pseudomonas fluorescens helA* mutant was significantly attenuated in root and shoot colonization of sugar beets (302).

A second subgroup consists of genes encoding the DNA-modifying enzymes DNA methylases. DNA methylation can play a role in gene regulation by inhibiting the interaction between regulatory proteins and their target DNA sequences (151). In several pathogens, altered DNA methylation patterns result in avirulent strains. Alteration of DNA methylation enables bacteria to control temporal gene expression in response to environmental stimuli and provides a mechanism by which information about environmental parameters experienced by parental cells can be inherited by the progeny cells (151).

Several genes are involved in DNA repair. Genes encoding the three subunits of the excision nuclease ABC (*uvrA*, *uvrB*, and *uvrC*) were isolated from three unrelated bacteria, *Ralstonia solanacearum* (26), *Porphyromonas gingivalis* (298), and *Mycobacterium tuberculosis* (56), respectively. Upregulated expression of the excision nuclease ABC during bacterial life in both animal and plant hosts suggests a major role for this repair enzyme during bacterial life in the host environment. Similarly, *recB* and *recD*, encoding subunits of exonuclease V,

were identified through IVET in *Yersinia enterocolitica* (300), and *Salmonella enterica* serovar Typhimurium (98), respectively. RecBCD is known to participate in the repair of double-strand breaks but was recently also implicated in the virulence of *Salmonella enterica* (33). It was demonstrated that RecBCD is required for infection of mice, since mutants lacking a functional RecBCD are avirulent in mice and are unable to grow in macrophages. It was suggested that systemic infection by *Salmonella enterica* may require RecBCD-mediated recombinational repair to prime DNA replication inside phagocytes (33).

In addition, it was demonstrated with IVET that the *Shigella flexneri alkA* gene is specifically upregulated during infection of mice (244). AlkA is a 3-methyladenine DNA glycosylase and is mainly involved in DNA damage repair but also functions in hypoxanthine excision and DNA demethylation (158, 213). In both mice and HeLa cell infection, a *Shigella flexneri alkA* mutant was significantly outcompeted by the wild type, suggesting a vital role for *alkA* in pathogenesis (244). The uracil DNA glycosylase encoded by *udg*, which was isolated from *Listeria monocytogenes* (295), might have a similar significance for macrophage infection. Udg is critical for removal of uracil resulting from cytosine deamination from DNA (203)

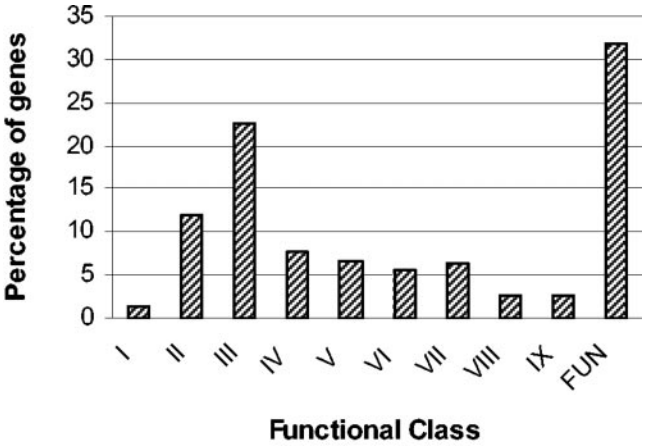

FIG. 3. Distribution of promoter trap-isolated host-induced genes among different functional classes. The percentages of genes involved in chemotaxis and motility (class I), nutrient scavenging (class II), central metabolism (class III), adaptation to environmental stresses (class IV), regulation (class V), cell envelope structure and modification (class VI), virulence and secretion (class VII), nucleic acid metabolism (class VIII), and transposition and site-specific recombination (class IX) and FUN genes (genes with unknown function or without significant similarity with known genes) are presented in the diagram.

Genes Involved in Transposition and Site-Specific Recombination

Table 2 shows that several transposases linked with insertion sequence elements, resolvases, and recombinases were found to be induced in host environments. For example, the *Salmonella enterica* serovar Typhimurium *gipA* gene, which shares homology with the IS*891*-like insertion element, was isolated with IVET (264). This family of insertion sequence elements is widely distributed among prokaryotes. Expression analysis showed that *gipA* is specifically induced in the early stages of infection. Consistent with the expression profile, it was demonstrated that a *gipA* mutant strain exhibits decreased survival in Peyer's patches after oral infection but not during infection of epithelial cells or spleen (264). This study clearly demonstrates that insertion sequence elements can play an important role in pathogenesis.

Insertion sequence elements are known to play a role in bacterial microevolution, but they might also play a general role in the modulation of gene expression of adjacent genes (183). We speculate that the genes belonging to this class might contribute to genetic variability for better adaptation and survival of bacterial species in a particular niche. In line with this, it was shown that transposition of the catabolic transposon Tn*4652* in nutrient-starved *Pseudomonas putida* (104) is triggered by environmental cues through a two-component system, ColR-ColS. This signal relay system was previously identified as an important root colonization factor in *Pseudomonas fluorescens* (49).

FUN Genes

Besides the extensive list of host-induced genes in Table 2, a considerable number of genes encoding putative proteins with unknown function or genes without significant homology to known proteins, referred to as FUN genes (101), were isolated with IVET and DFI. These FUN genes constitute almost a third of the total number of host-induced genes (Fig. 3), indicating that there is still a lot to be learned about bacterial life in natural environments.

Follow-up studies of some IVET- and DFI-isolated genes unequivocally demonstrated that FUN genes can be very important for bacterial life in the host environment. For instance, it was confirmed by mutational analysis that the DFI-isolated *Salmonella enterica* serovar Typhimurium *mig14* gene (279) is required for lethal infection of mice (277). Recently, it was shown that *mig14* encodes an inner membrane-associated protein that promotes persistent infection, survival within macrophages, and resistance to an antimicrobial peptide (23). IVET enabled the isolation of two other genes with unknown function, of which the corresponding mutants showed attenuated virulence in a mouse infection model: the *Vibrio cholerae iviXIII* gene and the *Staphylococcus aureus ivi17* gene (32, 152). FUN genes involved in interaction with plants have also been identified. A transcriptional fusion to the *Sinorhizobium meliloti nex4* gene, which revealed no similarity to known genes, was expressed in a restricted zone (nodule tip) of the alfalfa nodule. The *nex4* mutant was significantly attenuated in nodule formation and in its overall nitrogen-fixing capacity (188).

A considerable fraction of promoter trap-isolated FUN genes are predicted to be (integral) inner membrane proteins. DFI revealed the up-regulation of *ipc009* during *Streptococcus pneumoniae* infection of the murine respiratory tract. The *ipc009* gene encodes a predicted integral inner membrane protein. Analysis of a *Streptococcus pneumoniae ipc009* mutant revealed that a slightly higher dose was required to achieve the same lethality during infection of mice (160).

The frequent isolation of FUN genes that are specifically expressed in the host environment indicates that knowledge about the function of many genes in growth and survival in complex niches is still lacking and points to the need for further functional analysis of these genes to explore their exact role.

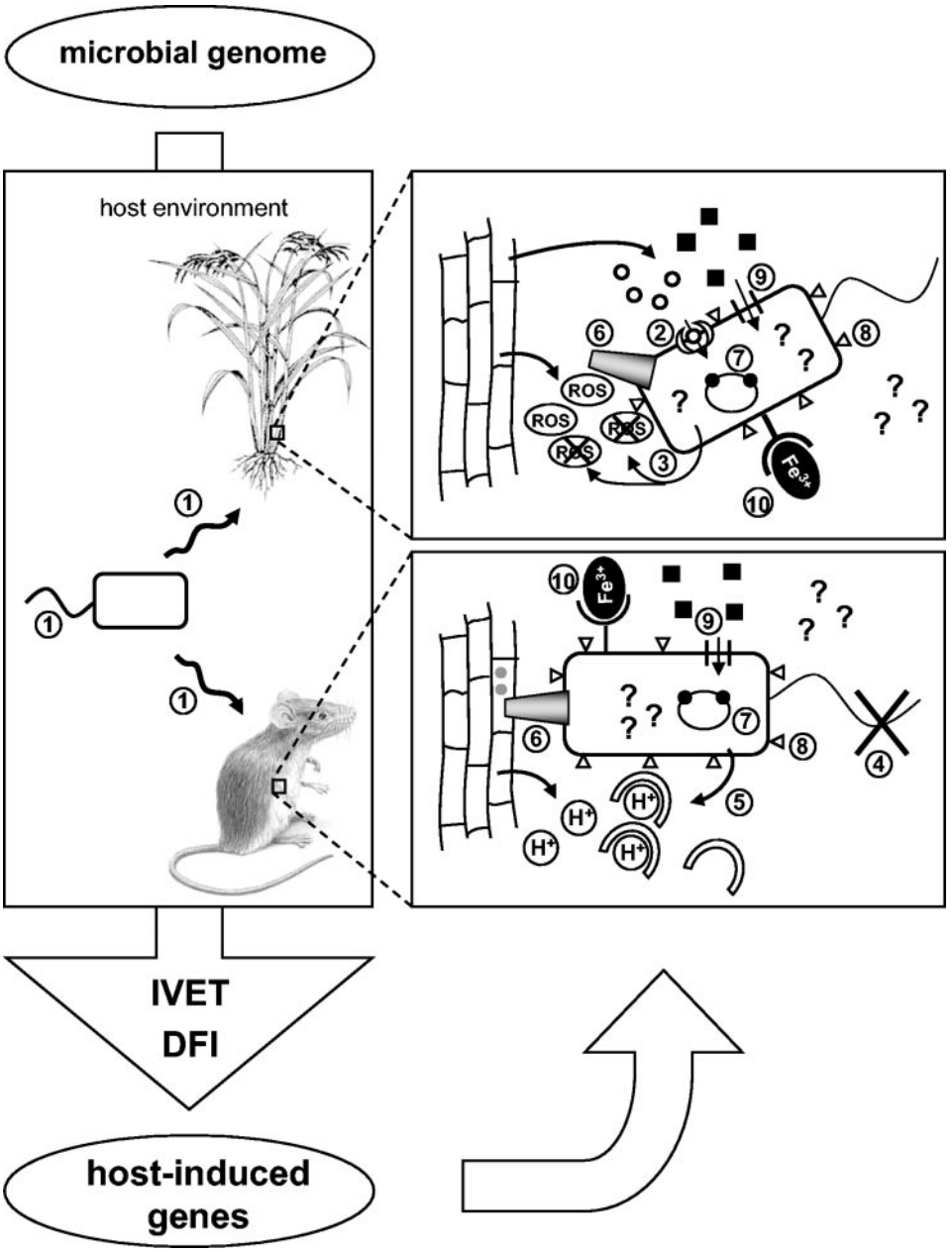

FIG. 4. Schematic overview of bacterial genes that are induced during life in the host environment. The host environment (black box) is a complex system of environmental parameters that activate or repress expression of several microbial genes. Activated genes can be isolated with promoter-trapping techniques such as IVET and DFI. Genes involved in chemotaxis and motility (1) are specifically expressed during (early stages of) interaction with the host. IVET and DFI studies of plant- and animal-associated microorganisms reveal several parallels and dissimilarities. Genes involved in amino acid uptake (2) and in direct inactivation of reactive oxygen species (3) were predominantly isolated from plant-associated microorganisms, while genes involved in suppression of chemotaxis and motility (4) and the acid stress response (5) were predominantly found in animal-associated microorganisms. In both ecological niches, genes involved in TTSS (6), DNA modification (7), cell surface modification (8), nutrient scavenging (9), and more specifically, iron acquisition (10) are upregulated. A significant number of the genes that are specifically induced in the wild have so far unknown functions (indicated with a question mark).

Several attempts are being made to unravel the function of FUN genes that are upregulated in the host environment.

CONCLUSIONS AND PERSPECTIVES

The establishment of a microorganism in a particular environment is a complex process requiring coordinated expression of many genes (Fig. 4). Gene expression needs to be modulated in response to changes in osmolarity and pH and availability of nutrients, just to mention a few of the environmental parameters that can change drastically and are experienced by the microorganism at various stages during niche colonization. This spatial and temporal fine-tuning implies the concomitant up- or downregulation of specific subsets of genes. Superimposed on this, for microbes interacting with a (eukaryotic) host, active communication to sustain the interaction also requires differential gene expression.

IVET: a Powerful and Flexible Tool

In vitro studies have their limitations in the study of host-microbe interactions. Many virulence genes are likely to remain unidentified because in vitro conditions cannot mimic all environmental cues that control gene expression. Therefore, a variety of genetic approaches (e.g., IVET, DFI, microarray, and SCOTS) have been developed. Opportunities to study microbial behavior in complex environments have thereby increased and have enabled the identification of novel traits that respond to environment-derived signals and may also contribute to ecological performance. Genes whose expression cannot be induced in vitro can be isolated with in vivo expression techniques.

Two main genetic approaches are used to study microbes in their natural habitat. One approach (exemplified by STM) is based upon inactivation of genes and subsequent phenotypic characterization of the mutants. However, relevant mutations may be overlooked during phenotypic screening because microbial physiological versatility often compensates for these defects. Furthermore, ecological performance is the result of coordinated expression of an ensemble of genes rather than being determined by single genes (143, 218). In a second approach, techniques are used that rely on analysis of gene expression patterns. The use of promoter traps (such as IVET and DFI) is based on the assumption that a major fraction of the genes that are important for bacterial life in complex environments are likely to be upregulated in these conditions.

Our survey indicates that subsets of STM- and IVET-isolated genes show little overlap, suggesting that these techniques are complementary (10). On the other hand, many genes isolated with SCOTS have also been identified with IVET or vice versa (44, 105). This is not surprising, as both techniques rely on the detection of differential gene expression, in contrast to STM.

With IVET, SCOTS, DFI, and STM, it is a laborious task to produce complete coverage of a genome, in contrast to the use of microarrays. Microarrays have the additional advantage that gene expression can be quantified. However, all techniques that are based upon isolation of microbial mRNA from a natural habitat (such as SCOTS or microarrays) experience difficulties due to the instability of RNA. Contamination originating from other microorganisms in the environment of interest raises additional problems. Also, the number of isolated microorganisms is often too small to isolate a sufficient amount of quality RNA (102). Microarrays are also limited because they measure the average of gene expression of the total bacterial population. Differences in gene expression occurring within a population residing in a heterogeneous environment may therefore be masked (20), whereas with IVET an individual bacterium expressing a gene in such an environment can be recovered. Furthermore, the construction of microarrays requires the availability of the annotated genome sequence of the organism under study. These requirements explain why microarray technology is mostly used to study differential gene expression of microorganisms cultured in defined media and in well-controlled environmental conditions. Nevertheless, microarrays were applied successfully to study bacterial gene expression in relatively simple habitats for which environmental parameters are easy to control on a laboratory scale (102, 241, 250), such as *Mycobacterium tuberculosis*-infected macrophages (270).

Global patterns of gene expression can reveal members of gene regulons. Cluster analysis of in vitro transcriptome profiles often proved useful to assign a possible function to genes of unknown function or to reveal regulatory networks (81). Valuable information about gene expression profiles in the wild can also be obtained through IVET. For instance, it was shown that approximately one third of all IVET-identified *Pseudomonas syringae* genes that are upregulated during *Arabidopsis thaliana* infection is under the control of the alternative sigma factor HrpL (16).

The power and the usefulness of IVET are apparent from the following: (i) many microbial IVET-isolated genes were already known to be involved in interaction with a host; (ii) induction of several host-induced genes was confirmed by independent IVET isolation of homologues in more than one microorganism; (iii) the upregulation of a variety of IVET-isolated genes was demonstrated with other techniques (SCOTS, reverse transcription-PCR, or microarray); (iv) in several cases, IVET isolation of a transcriptional fusion was followed by a spatiotemporal expression analysis by means of the reporter gene provided on the promoter trap, confirming the induction of gene expression in the wild; and (v) several IVET-isolated genes that were not known previously to be expressed in the conditions under study were analyzed further, and subsequent mutational analysis unequivocally demonstrated their ecological significance in the microbe's natural habitat.

Once the promoter trap library is constructed, it can be applied to study that particular microorganism in all sorts of complex environments. For instance, the IVET library constructed in *Streptococcus gordonii* was originally used to study gene expression during infection of mice (127). The same library was subsequently used to analyze gene expression during growth on saliva-coated hydroxyapatite (142) and during biofilm formation on polystyrene surfaces (304). Likewise, the IVET library constructed for the study of *Pseudomonas aeruginosa* infecting mice was subsequently used to study biofilm formation (67) and infection of burned tissues (92).

The same IVET library was used to identify *Yersinia enterocolitica hre* (host-responsive element) genes (300) and *sif* (systemic infection factor) genes (83). By altering the time of reisolating bacteria, virulence factors that are specifically expressed during the early stage of infection (*hre*) and genes required for systemic infection (*sif*) were isolated. When both subsets of IVET genes were compared, only *fyuA* (encoding the yersiniabactin siderophore receptor) was isolated in both screens, indicating that different subsets of genes are required at different stages of infection. Likewise, altering the mode of infection (intraperitoneal, ear cavity, or respiratory tract) revealed dissimilarities in the subsets of DFI-identified genes during *Streptococcus pneumoniae* infection of mice. Some genes were isolated with the three different infection methods, but various genes showed tissue-specific gene expression (160). This again points to the complexity of bacterial gene expression during interaction with a host and the need for appropriate tools to study this.

In addition to the isolation of genes induced in the wild, promoter trap strategies make it possible to subsequently in-

vestigate the expression of the captured genes in this complex environment by using the reporter gene provided on the promoter trap. In particular, with promoter trap strategies such as RIVET and DFI, it is possible to quantify the temporal and spatial expression of the genes of interest and analyze microenvironments in the host. For instance, RIVET enabled an in depth spatiotemporal expression analysis of secreted aspartic proteinases in *Candida albicans* (131, 261, 263). RIVET was also adapted to explore the complex gene expression patterns of known virulence factors (TcpA and CtxA) (7, 136, 139). RIVET application in *Vibrio cholerae* demonstrated that *tcpA* (encoding a pilin subunit of the toxin-coregulated pilus) and *ctxA* (encoding the enzymatic subunit of the cholera toxin) are not coregulated in the wild, in contrast to what was generally accepted (175). The versatility of RIVET is further illustrated by the design of a variant designated SIVET (for selectable in vivo expression technology) that enabled the quantitative analysis of dependence of prophage induction on host cellular physiology (147).

The knowledge gathered by IVET and DFI about the genes that are required for establishment of a pathogen in its host environment can be exploited for identifying new targets to direct new antimicrobial drug development or the construction of live attenuated vaccines (4, 235, 252). The subset of bacterial antigens that are highly expressed during infection can be rapidly evaluated for use in vaccine development (229). Hemolysin A and listeriolysin, for instance, are IVET-isolated genes which are highly suitable for the elicitation of cell-mediated immunity and might be used as tools for vaccine delivery (50).

The promoters that are isolated with IVET or DFI can also be used to drive spatially and/or temporally controlled heterologous gene expression, e.g., when the recombinant gene product is only desired at a specific stage during interaction with a host. Promoters that are specifically active in the rhizosphere can, for instance, be used to drive the conditional expression of genes involved in biocontrol.

The identification of genes induced in a particular environment provides information about the nature of the environment in which the organism functions and how the organism perceives its environment. For instance, genes involved in resistance to acid stress were only isolated from animal pathogens, indicating that plant-associated bacteria are less exposed to this type of stress.

Concluding Remarks

In many studies, the impetus for the development and application of promoter trap technologies has been to obtain insights into the mechanistic bases of bacterial fitness in complex environments: genes specifically activated in the wild are likely to contribute to the ecological success of the organism in that environment. Explicit tests of this hypothesis, which involves the construction of mutants and analysis of their performance in the environment of interest, are relatively few compared to the large number of genes identified. To cite only a few representative studies, Camilli and Mekalanos (32), Valdivia and Falkow (279), and Gal et al. (78) constructed mutations in genes showing elevated levels of expression in the mouse intestine for *Vibrio cholerae*, in macrophages for *Salmonella enterica* serovar Typhimurium, and in the sugar beet rhizosphere for *Pseudomonas fluorescens*, respectively, and found that these mutants were compromised in their ability to grow in their host environments. However, in several other instances where mutants defective in IVET- or DFI-identified genes were constructed, such phenotypes were not revealed. This was, for instance, the case with a TTSS mutant of sugar beet-colonizing *Pseudomonas fluorescens* (212).

The reasons for nondefective phenotypes are numerous and could indicate that (i) the gene of interest (and the trait toward which it contributes) has no ecological significance; (ii) there is genetic redundancy; (iii) the gene is ecologically significant but it contributes subtly, in a quantitative manner, to the trait in question, and as a consequence defective phenotypes are not generated; and (iv) the gene does contribute significantly to ecological performance but assays of fitness inadequately account for environmental complexity and thus return spurious results.

To expand on this last point, imagine the following hypothetical scenario. Gene X contributes significantly to ecological performance but only in niches where the inducer of gene X is present. In the absence of knowledge about the spatial and temporal distribution of the inducing signal, a gene X mutant is made to compete against the wild type. After a period of time the ratio of mutant to wild type is determined, and no significant difference is found. Unbeknownst to the experimenters, though, is that only 10% of the bacteria colonized niches where the inducing signal was present, and despite a significant reduction in fitness, the effect was masked by the 90% of cells that grew in niches where gene X was not induced. This plausible scenario draws attention to the fact that a valid measure of the fitness effects of specific genes is dependent upon an understanding of the spatial and temporal distribution of inducing signals in the environment of interest.

The importance of the ability to adapt to the changing nutritional environment is reflected in the high proportion of trapped genes that are involved in metabolism (Fig. 3). These genes are often considered of less interest because they are linked to housekeeping functions and do not encode "genuine" colonization or virulence factors. However, since metabolic genes are isolated frequently in practically all IVET studies, their increased expression tells us something: getting food matters! The identification of metabolic genes specifically expressed in these conditions can render new information about the metabolism of the microbe that may, in the case of animal pathogens, prove useful for vaccine development.

Our overview reveals that microorganisms display niche-specific gene expression rather than an expression pattern that is common to phylogenetically related species. Niche-specific gene expression is not surprising, since several microorganisms seem to possess similar mechanism for acclimation, protection, and survival in the host environment. Moreover, they are exposed to similar environmental challenges and have to change their gene expression in response to the same environmental stimuli.

Evidence has accumulated in recent years that plant and animal pathogens share more features than previously recognized. For instance, gram-negative bacterial pathogens use a common machinery (TTSS) to deliver effector proteins into eukaryotic cells (31). The legume symbiont genus *Rhizobium*,

the phytopathogen genus *Agrobacterium*, and the animal pathogen genus *Brucella* use similar strategies to achieve host infection (202). Certain bacteria, such as *Pseudomonas aeruginosa*, are capable of infecting plants and animals using overlapping virulence mechanisms (217). More such parallels have appeared from studies of gene expression in the wild, such as iron acquisition, nutrient metabolism, stress response, and glutathione-mediated protection against oxidative stress. This reflects the use of antimicrobial protection strategies that are shared by animal and plant hosts, and these conserved defense mechanisms apparently elicit similar responses in the invading microorganisms (12, 103).

IVET also revealed unexpected parallels between pathogens and nonpathogens, such as the expression of a TTSS in the nonpathogenic *Pseudomonas fluorescens* during sugar beet colonization (212, 218) and the IVET identification of an *Agrobacterium tumefaciens* ChvD homologue in *Salmonella enterica* serovar Typhimurium which is normally required for plant pathogenesis. Promoter traps also revealed that a common set of genes are upregulated in animal pathogens and in bacteria that are assumed to be beneficia; to the animal hosts, such as some *Lactobacillus* species. Such information should be taken into account when developing new antibiotics or therapies in order to avoid collateral damage to beneficial bacteria.

Camilli and Mekalanos (32) were the first to discuss the isolation of transcriptional fusions from *Vibrio cholerae* that are apparently oriented in the wrong direction to drive reporter gene expression. Since then, several apparent antisense transcripts were identified with IVET from *Pseudomonas aeruginosa* (290), *Pseudomonas fluorescens* (218), *Pseudomonas stutzeri* (224), *Ralstonia solanacearum* (26), *Histoplasma capsulatum* (226), *Porphyromonas gingivalis* (298), *Listeria monocytogenes* (55), and *Staphylococcus aureus* (152). At present, very little is known about these putative antisense transcripts, but considering the frequency at which antisense transcripts are identified with promoter traps, it is likely that they have a real function rather than being artifacts of the technique. They might be involved in down-regulating gene expression (32, 175, 246).

The corresponding host-induced promoters might drive the expression of small noncoding RNA molecules (246). Recent genomewide screens resulted in the identification of approximately 50 small RNAs in *Escherichia coli*, suggesting that they occur more abundantly than expected (225). Small RNAs are involved in the regulation of protein synthesis by affecting transcription (by pairing with their target mRNAs), translation, and stability. In addition, by binding to proteins, small RNAs can alter their activity. Many noncoding RNAs require the RNA-binding protein Hfq for activity. Interestingly, the *Pseudomonas stutzeri* A15 *hfq* gene was also found to be induced in the rice rhizosphere (224). These observations are in agreement with the idea that small noncoding RNA molecules are important for environmental adaptation (225). Notably, IVET enabled the identification of an RNase acting specifically on small oligoribonucleotides that is upregulated in *Pseudomonas fluorescens* during sugar beet colonization (303).

In recent years, DNA sequence data for entire microbial genomes have accumulated rapidly. But since it is impossible to assign a biochemical and biological function for many genes based purely on annotation and comparative genomics, the need to perform additional mutational and expression analyses, that is, functional genomics, remains. However, these experiments are hampered by the complexity of the natural environment of the microorganisms. Promoter traps, IVET in particular, are powerful tools for high-throughput screening for genes that are specifically expressed in these complex environments. Considering the continuous flow of new IVET papers, it appears that the IVET star is still rising and will continue to render new insights into the secret lives of bacteria.

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