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16. The 1.2-kb Hpa I fragment of the *RAS2* gene was cloned into a Hinc II cleavage site of M13tv18 phage DNA (Takara Shuzo, Kyoto, Japan) and subjected to oligonucleotide-directed mutagenesis with the gapped duplex method [W. Kramer and H.-J. Fritz, *Methods Enzymol.* **154**, 350 (1987)]. We used oligonucleotides GGGTGGCTCTTGTATT and TGGCTGTTCTATTATAAG to introduce substitutions of single amino acids of serine for Cys^{318} or Cys^{319}, respectively. The *RAS2* gene fragments carrying the mutations were transferred to *pBlueBac-βL1*, and we used the resulting plasmids to produce recombinant viruses (*14*). The intermediate form was purified as described (*15*). The unprocessed form was extracted without the detergent and purified in a similar manner.
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18. The structure of *YEP24-ADC1-CYR1*, which contains the complete wild-type *CYR1* gene under the yeast alcohol dehydrogenase I (*ADC1*) promoter, has been described (*19*). The plasmid was transformed into yeast strain TK35-1 (*MATα, leu2, his3, trp1, ura3, cyr1-2,* and *ras2::LEU2*) with Li acetate [H. Ito, Y. Fukuda, K. Murata, A. Kimura, *J. Bacteriol.* **153**, 163 (1983)]. A crude membrane fraction was prepared from yeast cells (*13, 19*). The adenylyl cyclase assay was performed as described (*4, 13*), except that Ras2 was incubated with 0.1 mM GTP-γ-S for 30 min at 30°C in 20 mM tris-HCl (pH 7.4), 10 mM EDTA, 1 mM DTT, and 5 mM $MgCl_2$ before it was added to the reaction mixture. Crude membrane (11.6 μg) having 100 units [1 unit = 1 pmole of adenosine 3′,5′-monophosphate (cAMP) production per minute per milligram of protein] of Mn^{2+}-dependent adenylyl cyclase activity (*4, 13*) was assayed in a reaction mixture (100 μl) containing various concentrations of the preincubated Ras2 protein. We quantitated the three forms of Ras2 protein by measuring the saturated amounts of bound [^{35}S]GTP-γ-S.
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21. We prepared a polyclonal antiserum to Ras2 by immunizing rabbits with the Ras2 protein produced in *E. coli* as a fusion protein with the *Schistosoma japonicum* GST and purified by glutathione-agarose chromatography as described [D. B. Smith and K. S. Johnson, *Gene* **67**, 31 (1988)]. All procedures with animals were performed in accordance with institutional guidelines. Ras2 protein was detected by protein immunoblotting as described [H. Towbin, T. Staehlin, J. Gordon, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4350 (1979)].
22. A segment of the *CYR1* gene corresponding to amino acid positions 606 to 2026 was fused to the COOH-terminus of GST and overexpressed in yeast cells under the *ADC1* promoter in yeast expression plasmid pAD1 (*13*). Adenylyl cyclase complex containing the GST-*CYR1* gene fusion product was solubilized by 1.3 M NaCl (*20*) and purified by glutathione-agarose chromatography (*21*). After elution with 5 mM glutathione in 50 mM tris-HCl (pH 8.0) and 0.15 M NaCl, adenylyl cyclase complex was separated from glutathione by chromatography (Sephadex G-25, Pharmacia) and used for the assay.
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Selection of Bacterial Virulence Genes That Are Specifically Induced in Host Tissues

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A genetic system was devised that positively selects for bacterial genes that are specifically induced when bacteria infect their host. With the pathogen *Salmonella typhimurium*, the genes identified by this selection show a marked induction in bacteria recovered from mouse spleen. Mutations in all *ivi* (in vivo–induced) genes that were tested conferred a defect in virulence. This genetic system was designed to be of general use in a wide variety of bacterial-host systems and has several applications in both vaccine and antimicrobial drug development.

To understand the mechanisms by which a bacterial pathogen circumvents the immune system and causes disease, one has to identify those bacterial gene products that are specifically required for each stage of the infection process. The expression of most known virulence factors is regulated by environmental conditions in vitro that presumably reflect similar cues present in host tissues (*1*). The identification of many virulence factors has been dependent on, and limited by, the ability to mimic host environmental factors in the laboratory. We have developed a genetic system, termed IVET (in vivo expression technology), that does not rely on the reproduction of these environmental signals but rather depends on the induction of genes in the host. Thus, we selected bacterial genes that are highly expressed in host tissue but minimally expressed on normal laboratory media. Presumably, a subclass of genes that meet these selection criteria will encode products required for the infection process, including previously unidentified virulence factors.

The IVET strategy begins with a bacterial strain carrying a mutation in a biosynthetic gene that greatly attenuates growth in vivo; in this case, we used a *purA* auxotroph of *Salmonella typhimurium* (*2*). Growth in the host of such a mutant strain is then complemented by operon fusions to the same biosynthetic gene. The efficient selection for a $PurA^+$ phenotype in animal tissues (>10^8-fold selection) provides a means of positive selection for bacterial promoters that are expressed in host tissues by simply fusing them to a promoterless *purA* gene. In devising the system, we met three criteria: (i) construction of the fusions in single copy on the bacterial chromosome (to avoid complications that arise from using plasmids); (ii) no disruption of any chromosomal genes (to avoid the loss of any potential virulence factors); and (iii) provision of a convenient method to monitor the transcriptional activity of any given fusion both in vivo and in vitro.

We accomplished these goals by first constructing a synthetic operon consisting of a promoterless *purA* gene and a promoterless *lac* operon cloned into the broad host range suicide vector, pGP704 (*3*), resulting in pIVET1 (Fig. 1). The cloning of Sau 3AI *S. typhimurium* chromosomal fragments into the Bgl II site 5′ to the *purA* gene resulted in the construction of transcriptional fusions, where properly positioned *S. typhimurium* promoters drive the transcription of wild-type copies of *purA* and *lacZY*. Introduction of this pool of fusions into a Δ*purA* strain of *S. typhimurium* (*4, 5*) and selection for ampicillin resistance required the integration of the recombinant plasmids into the chromosome by homologous recombination with the cloned *S. typhimurium* DNA (Fig. 1). The product of this integration event generated a duplication of *S. typhimurium* DNA in which the native chromosomal promoter drove the *purA-lac* fusion, whereas the cloned promoter drove the expression of a wild-type copy of the gene.

The pattern of *lacZ* expression in a random pool of fusions was assessed in vitro on MacConkey Lactose indicator medium.

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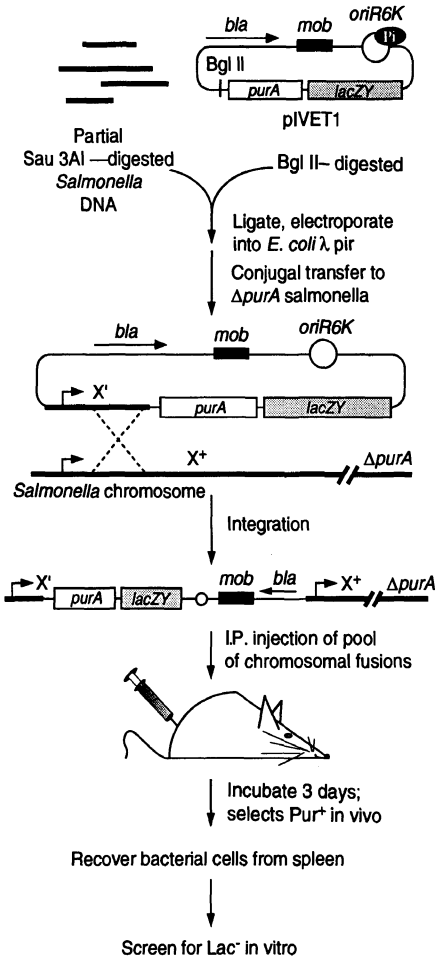

Fig. 1. Positive selection for *ivi* genes that are specifically induced in the host. See text for details.

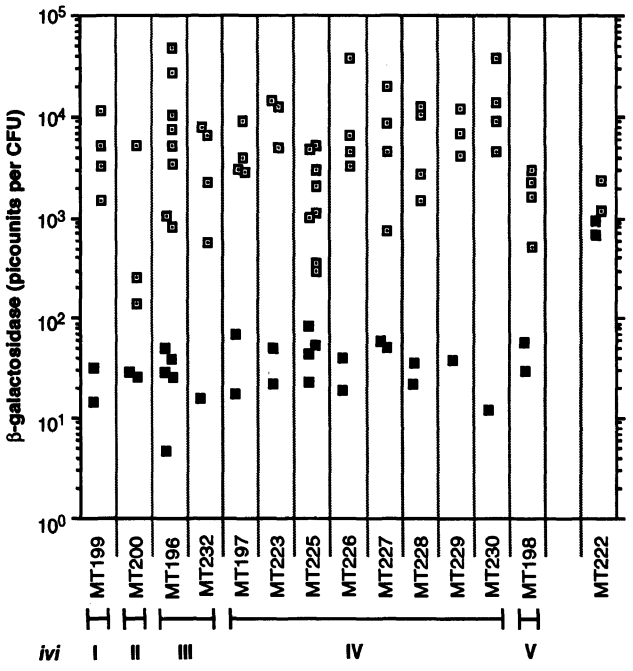

Fig. 2. β-galactosidase expression from bacterial cells that were recovered from mouse spleen versus the same strain grown on rich media. The vertical axis depicts the picounits of β-gal per colony-forming unit, where units of β-gal equal micromoles of *o*-nitrophenol (ONP) formed per minute. The open boxes denote the β-gal activity of cells recovered from the spleen (*26*). The closed boxes denote the β-gal activity from cells grown overnight in rich medium (LB). Individual strains are grouped according to their respective *ivi* fusions.

The level of *lacZ* expression required to give a Lac$^+$ phenotype appeared to correspond to the level of *purA* expression required to supplement the parental PurA auxotrophy; that is, colonies that were Lac$^+$ (red) on MacConkey Lactose indicator medium were Pur$^+$ on minimal medium; colonies that were Lac$^\pm$ (pink) were semi-auxotrophic on minimal medium; and colonies that were Lac$^-$ (white) were Pur$^-$ on minimal medium. Of the preselected fusion strains, 33% (116/346) were Lac$^+$, 16% (56/346) were Lac$^\pm$, and 50% (174/346) were Lac$^-$. This indicates that before selection in the mouse, only 33% of the fusion strains displayed sufficient in vitro expression to result in both a Lac$^+$ and a Pur$^+$ phenotype.

The pool of integrated *purA-lac* fusion strains (10^6 organisms) was injected intraperitoneally into a BALB/c mouse. After 3 days, bacterial cells were recovered from the spleen, and these cells were injected into a second mouse, where the process was repeated. Only bacteria that expressed *purA* at a high enough level to overcome the parental purine deficiency should have survived and multiplied in the mouse. Indeed, bacterial cells recovered from the spleen had an increased percentage of cells that were Lac$^+$ (and therefore PurA$^+$) compared to the initial inoculum: 86% (235/273) of the bacterial cells recovered from the spleen were Lac$^+$, 9% (24/273) were Lac$^\pm$, and 5% (14/273) were Lac$^-$. Such a shift toward Lac$^+$ cells was consistent with the selection of strains that contain fusions to promoters that are transcriptionally active in vivo. We focused our subsequent analysis on the 5% Lac$^-$ class of fusion strains. Although these strains had sufficient *purA* transcription to survive within the mouse, they showed little expression on laboratory media; therefore, these strains contain fusions to genes [termed *ivi* (in vivo–induced] that are specifically induced in the mouse. Fifteen of these Lac$^-$ strains were chosen at random for further study.

To confirm that these fusions were specifically induced in vivo, we directly assayed the β-galactosidase (β-gal) activity in bacteria recovered from spleens of infected mice and compared this activity to that measured for the same strain grown overnight in rich medium (Fig. 2). Although there was considerable mouse to mouse variability with any given fusion strain tested, in every case the fusion strains recovered from the spleens were induced in β-gal activity that was 40- to 1000-fold greater than that of the same strain grown in rich medium. As a control for this experiment, we chose, from our initial unselected pool, a random Lac$^+$ fusion strain, MT222, that had high β-gal activity on laboratory media. This random nonselected fusion was not significantly induced in the mouse as compared to β-gal production after growth on rich medium; the β-gal activity was relatively high in both conditions.

We have used a genetic approach to clone the 15 selected *ivi* fusions directly from the bacterial chromosome by phage P22 transduction (*6*). Using a primer homologous to the 5′ end of the *purA* gene, we sequenced approximately 200 to 400 base pairs of *S. typhimurium* DNA immediately upstream of the *purA* gene in each of the cloned fusions. Sequence analysis indicates that the 15 fusions represent five different genes. Two of five fusions (*iviIV* and *iviV*) are in genes that show no significant homology to sequences in GenBank (*7*) (Genbank version 72). This suggests that the IVET system has identified previously uncharacterized genes that are specifically induced during mouse infection.

One fusion to a known sequence was to the *carAB* operon (*ivil*), whose genes encode the two subunits of carbamoyl-phosphate synthetase. This enzyme is involved in arginine and pyrimidine biosynthesis (*8–10*). The induction of this operon is consistent with the apparent low availability of pyrimidines in animal tissues (*11*). Thus, the IVET selection allows the identification of those products involved in intermediary metabolism and whose induction in vivo is necessary for virulence and survival in animal tissues.

The second fusion is located in the *pheST himA* operon (*iviII*) that encodes the two subunits of phenylalanyl-tRNA synthetase and one subunit of integration host factor (IHF), which is a DNA binding protein involved in DNA replication, gene regulation, and site-specific DNA recombination (*12–14*). It is not known why the genes for phenylalanyl-tRNA synthetase and a subunit of IHF are transcribed together; therefore, it is not clear whether induction of this operon in vivo is in response to a depletion in charged tRNA or a demand for an increased level of IHF for some regulatory function. Changes in type I pilin expression,

Table 1. Mutations in operons defined by the *purA-lac* fusions confer a virulence defect.

Bacterial strain*	Relevant genotype†	Oral LD_{50}‡
14028	Wild type	10^4
MT219	*ivil-6*::Mud-Cm (*carAB*)	$>2\times10^8$
MT220	*iviII-7*::Mud-Cm (*pheST himA*)	$>2\times10^8$
MT221	*iviIV-9*::Mud-Cm	2×10^6

*All bacterial strains used in this study are derivatives of *S. typhimurium* ATCC 14028 (CDC 6516-60). Mutant strains are isogenic to wild type except for a Mud-Cm insertion element in an *ivi* operon defined by its respective *purA-lac* fusion. †Genes in parentheses indicate the *ivi* operon whose expression is affected by the Mud-Cm insertion mutation. Mud-Cm insertion elements are defective Mu phages that contains a chloramphenicol resistance determinant (T. Elliot); transpositions of this element were done according to methods described previously (*27*). ‡For each of the mutant strains, groups of five mice were perorally infected with a dose of 10^6, 10^7, or 10^8 organisms. The dose required to kill 50% of infected animals (LD_{50}) for each of these strains was compared to that of the wild type.

dependent on IHF (*15*), do confer an advantage to *S. typhimurium* in preventing clearance from animal tissues (*16, 17*).

The third known fusion is in the *rfb* operon, which encodes approximately 20 genes involved in O-antigen synthesis, the outermost layer of lipopolysaccharide (*18, 19*). This fusion is located in the penultimate gene of the operon. However, the direction of transcription is in the opposite orientation to that of the *rfb* operon and would generate an antisense transcript to the *rfb* operon complementary to at least the 3′ end of the *rfb* mRNA. We are investigating whether this *ivi* transcript may act through an antisense mechanism to down-regulate O-antigen synthesis in vivo. Mutants defective in O-antigen synthesis are highly attenuated when delivered orally but are fully virulent when delivered intraperitoneally (*20*).

To determine the overall contribution of *ivi* genes to *S. typhimurium* pathogenesis, we have constructed mutant strains defective in *ivi* expression. Using the sensitive chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) in association with transposon mutagenesis, we have isolated Mud-Cm insertion elements in the cloned *ivi* operons. These transposons disrupt the gene at the site of insertion and furthermore reduce the expression of downstream genes in the same operon by polarity. Thus, the insertions decrease the expression of *lacZ*, shifting the colony color from blue (higher levels of expression) to light blue (lower levels of expression) on medium containing X-gal.

We determined the effect of reduced *ivi* expression on virulence by crossing the insertion mutations into an otherwise wild-type chromosome and orally challenging BALB/c mice with the resultant mutant strains. To date, we have been successful in constructing such insertion-bearing strains in three of five cases. The oral lethal dose required to kill 50% of infected animals (LD_{50}) for wild-type infection of a BALB/c mouse is 10^4 cells. Insertions in all three operons tested caused an increase in the LD_{50} from 200- to $>2 \times 10^4$-fold (Table 1). This indicates that our genetic system selects for genes that are important for *S. typhimurium* virulence. Furthermore, we tested whether mice immunized with these attenuated strains were resistant to oral challenge with wild-type *S. typhimurium*. Six out of thirteen mice previously orally immunized with MT220 (*iviII-7*::Mud-Cm) survived an oral challenge of wild-type organisms at a dose 10^5-fold above the LD_{50}, whereas 0 out of 15 control mice survived a challenge at this dose. This result suggests that the in vivo selection described here also provides a means of isolating attenuated mutants that may have utility as live vaccine strains.

Because purine auxotrophy attenuates many pathogenic bacteria (*2, 21–24*), the IVET selection system described here should be of general use in a variety of bacterial-host systems. Also, several other biosynthetic genes can in theory be used in this selection scheme. For example, we have constructed a *thyA-lac* fusion vector, pIVET2, for use in bacterial species where thymine auxotrophy results in attenuation (*21, 25*). One advantage of the *thyA* system is its ability to select for $thyA^-$ mutants with the antibiotic trimethoprim. Thus, the *thyA* system allows positive selection for fusion to genes that are both induced in vivo (by selecting Thy^+) and transcriptionally inactive in vitro (by selecting Thy^-).

The IVET system was designed to facilitate the identification of virulence factors and thus may contribute to vaccine and antimicrobial drug development. For example, *ivi* genes may encode new antigens, and mutations in *ivi* genes may provide an additional means of constructing live attenuated vaccines. It is expected that *ivi* promoters will be tested for possible use in obtaining expression of heterologous antigens in live attenuated vaccines. The elucidation of *ivi* metabolic gene products may also provide new targets for antimicrobial drug development.

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