Proc. Natl. Acad. Sci. USA
Vol. 94, pp. 934–939, February 1997
Genetics

Bacterial infection as assessed by *in vivo* gene expression

(pathogenesis/*in vivo* expression technology/*ivi*/*phoPQ*/*Salmonella*)

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Communicated by Robert L. Sinsheimer, University of California, Santa Barbara, CA, December 2, 1996 (received for review September 11, 1996)

ABSTRACT ***In vivo* expression technology (IVET) has been used to identify >100 *Salmonella typhimurium* genes that are specifically expressed during infection of BALB/c mice and/or murine cultured macrophages. Induction of these genes is shown to be required for survival in the animal under conditions of the IVET selection. One class of *in vivo* induced (*ivi*) genes, *iviVI-A* and *iviVI-B*, constitute an operon that resides in a region of the *Salmonella* genome with low G+C content and presumably has been acquired by horizontal transfer. These *ivi* genes encode predicted proteins that are similar to adhesins and invasins from prokaryotic and eukaryotic pathogens (*Escherichia coli* [*tia*], *Plasmodium falciparum* [PfEMP1]) and have coopted the PhoPQ regulatory circuitry of *Salmonella* virulence genes. Examination of the *in vivo* induction profile indicates (*i*) many *ivi* genes encode regulatory functions (e.g., *phoPQ* and *pmrAB*) that serve to enhance the sensitivity and amplitude of virulence gene expression (e.g., *spvB*); (*ii*) the biochemical function of many metabolic genes may not represent their sole contribution to virulence; (*iii*) the host ecology can be inferred from the biochemical functions of *ivi* genes; and (*iv*) nutrient limitation plays a dual signaling role in pathogenesis: to induce metabolic functions that complement host nutritional deficiencies and to induce virulence functions required for immediate survival and spread to subsequent host sites.**

Microbial pathogenicity may be defined by the ability to propagate and persist at sites in the host that are inaccessible to commensal species (1). Many virulence determinants that contribute to this ability share a unique phenotype: induction in the host. Previously, we have established a genetic approach, termed *in vivo* expression technology (IVET), which uses the animal as a selective medium to identify bacterial genes specifically induced during infection (2, 3). These *in vivo* induced (*ivi*) genes were shown to be poorly expressed on laboratory medium but exhibit relatively elevated levels of expression in host tissues or in cultured macrophages. It is not anticipated that all *ivi* genes will have an essential role in virulence. However, their *in vivo* induction suggests that they contribute to growth in restricted host tissues and thus enhance pathogenicity.

The IVET selection is a promoter trap, whereby bacterial promoters are selected that drive the expression of a gene that is required for virulence (Fig. 1). A promoterless *lacZ* gene is fused downstream of the promoterless *purA* gene to monitor the expression of the entire synthetic operon when cells are grown on laboratory medium or in animal tissues. Two variations of the IVET selection strategy have been employed, using *purA* and *cat* (chloramphenicol acetyltransferase) as the *in vivo*-selected markers (2, 3). A positive selection for *ivi* genes is provided by the need to complement a *purA* nutritional deficiency or to express *cat* in response to a host drug regimen. Here we show that induction of *ivi* genes is required for survival in the animal under the conditions of the IVET selection. This collection of *ivi* genes comprises an *in vivo* induction profile that reveals a broad array of regulatory, metabolic, and virulence functions that contribute to enhanced growth and persistence in host tissues.

MATERIALS AND METHODS

Media. Laboratory media used in these studies included Luria broth (LB) (4) and lactose MacConkey indicator medium prepared by adding 1% filter-sterilized lactose (Baker) to previously autoclaved MacConkey agar base (Difco). Final concentrations of antibiotics (Sigma) were as follows: ampicillin, 50 μg/ml; tetracycline, 20 μg/ml; and chloramphenicol, 20 μg/ml, unless otherwise designated. LB and MacConkey medium were supplemented with adenine (13.5 mg/ml) for the growth of *purA* strains.

Bacterial Strains and Phage. All *Salmonella typhimurium* strains used in this study were derived from strain ATCC 14028 (CDC 6516–60). The high-frequency generalized transducing bacteriophage P22 mutant HT 105/1, *int-201*, was used for all transductional crosses (5), and phage-free phage-sensitive transductants were isolated as previously described (6). Strains used for PhoPQ regulation studies were constructed by transduction of the IVET-selected fusion into ATCC 14028 (wild type), and isogenic *phoPQ* derivatives, KK16 [*phoP102*::Tn*10d*-Cm (7)] and KK64 [*phoQ24* (8)] kindly provided by Karl Klose (Harvard Medical School, Boston).

Cell Culture. The murine macrophage cell line RAW 264.7 was obtained from the American Type Culture Collection (ATCC TIB-71) and maintained in minimal essential medium (MEM) supplemented with Earle's salts, 10% heat-inactivated fetal calf serum, and 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (Hepes), pH 7.0. Cells were grown in a humidified atmosphere of 5% carbon dioxide and 95% air at 37°C in 75-cm^2 plastic flasks (Corning). For experiments, cells were harvested by scraping with a rubber policeman and were plated at a density of 1×10^6 cells per ml in 35-mm diameter, six-well dishes (Corning) and grown 24 hr to approximately 80–90% confluence ($2–5 \times 10^6$ cells per well) (9).

Construction of IVET Fusion Pools. pIVET1 and pIVET8 pools were made as described previously (2, 3) with the following modifications. *Sau*3AI partial restriction digests of *Salmonella* DNA were size fractionated (1–4 kb) on a 0.8% agarose gel. Tn*10d*-Tc insertion mutations were used to (*i*) provide a counterselectable marker for the introduction of the recombinant pools from *E. coli* into *Salmonella*, and (*ii*) create a nutritional deficiency (*purA*) that will be the basis for the *in vivo* selection. Thus, all pIVET1-containing strains harbor a *purA3131*::Tn*10d*Tc insertion mutation; all pIVET8-containing strains harbor a *zjf-7501*::Tn*10d*-Tc insertion mutation.

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0027-8424/97/94934-6$2.00/0
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Abbreviations: IVET, *in vivo* expression technology; i.g., intragastric(ally); DBL, Duffy binding-like; ORF, open reading frame.

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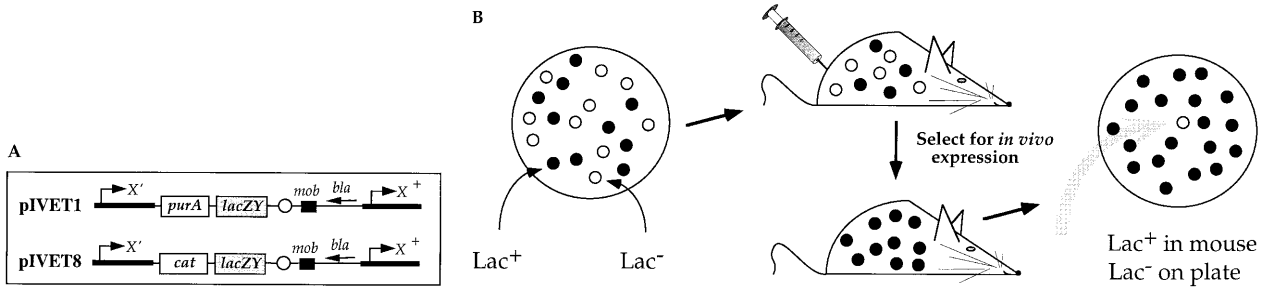

FIG. 1. Selection for bacterial genes that are specifically induced during infection. (*A*) Random fragments of bacterial DNA (dark arrows) were cloned into an IVET vector, 5′ to a promoterless *purA* or *cat* gene (2, 3). (*B*) The recombinant pool was used as an inoculum for infection of BALB/c mice and/or RAW 264.7 cultured macrophages. Bacterial survival in the animal (or cultured macrophage) is dependent on *in vivo* selection of bacterial promoters that drive the expression of the promoterless *purA* or *cat* genes. After incubation in the animal (or cultured macrophage), bacterial fusion strains were recovered from host tissues and plated on lactose MacConkey indicator medium. The gray arrow indicates an *ivi* fusion-bearing strain which is Lac^+ (ferments lactose) (●) when grown in the animal and Lac^- (○) when grown on laboratory medium.

IVET Selection in Cultured Macrophages. One hundred microliters of the *S. typhimurium cat-lac* fusion pools (5×10^8 cells) grown overnight in LB containing ampicillin and tetracycline was added to 5×10^7 RAW 264.7 macrophages that had been washed twice with cell-culture medium. After a 3-hr incubation, the coculture was washed four times with cell-culture medium and incubated for 2 hr in the presence of 100 μg/ml gentamicin to kill extracellular bacteria (10). These cells were washed four times with cell-culture medium and incubated overnight with 5 μg/ml gentamicin and 20 μg/ml chloramphenicol. The overnight coculture was washed three times with cell-culture medium, and the surviving intracellular bacteria were recovered by lysing the macrophages in 1 ml of distilled deionized H_2O. The recovered bacterial cells were grown overnight in LB containing ampicillin and tetracycline and used as inoculum for a second round of macrophage selection.

IVET Selection in BALB/c Mice. *Intragastric inoculation*. Pooled *ivi* fusions were grown overnight in LB, serially diluted to 10^8-10^9 cells, and used to i.g. (intragastrically) infect BALB/c mice. Bacteria were recovered from the small intestine after either 24- or 48-hr incubation or, in independent experiments, bacteria were recovered from the spleen or liver after mice showed visible signs of illness (6 days). The recovered bacterial cells were grown overnight in LB containing ampicillin and tetracycline and used as inoculum for a second round of selection.

Intraperitoneal infection. Pooled *ivi* fusions were grown overnight in LB, serially diluted to 5×10^5 cells, and used to intraperitoneally (i.p.) infect BALB/c mice. Bacteria were recovered from the spleen after mice showed visible signs of illness (3 days). The recovered bacterial cells were grown overnight in LB containing ampicillin and tetracycline and used as inoculum for a second round of selection. Mice inoculated i.p. with *cat-lac* fusions were administered chloramphenicol as previously described (3).

Cloning and Sequencing IVET Fusions. *ivi* fusion strains were cloned by transduction as described previously (11) or by triparental mating (P. Rainey, D.M.H., and M.J.M,, unpublished work). *ivi* fusion junctions (200–400 bp) were sequenced by using the following primers that are homologous to the 5′ end of the selected gene reading upstream into *S. typhimurium* sequence: pIVET1 (*purA*) [5′-CATTGGGTGCCCAGTACG-3′] and pIVET8 (*cat*) [5′-CAACGGTGGTATATCCAG-3′].

RESULTS

Selection of *in Vivo* Induced Genes. Following implementation of the pIVET1 (*purA*) and pIVET8 (*cat*) selection strategies in BALB/c mice and/or RAW 264.7 cultured macrophages, the recovered bacterial cells were plated on lactose MacConkey indicator medium. Both selections resulted in an enrichment in the fraction of Lac^+ clones in the postselected pool of fusions (red or pink colonies) compared with the preselected pool, indicating that transcriptionally active promoters were selected *in vivo*, as has been shown previously (refs. 2 and 3; Fig. 1). For example, the preselected pIVET1 pool consists of 48% Lac^+ and 52% Lac^- colonies. After separate i.g. infections, the ratio of Lac^+ to Lac^- colonies increased to 99:1 and 97:3 among bacteria recovered from the intestine or the spleen, respectively. This indicates that there is a mucosal and systemic purine deficiency and thus IVET-selected promoters are required at both early and late stages of infection under these conditions. Each IVET selection resulted in a similar enrichment (data not shown).

From 212,000 IVET-selected colonies plated, 2647 *ivi* fusions (Lac^-) were isolated, cloned, and restriction mapped. Siblings were identified by identical restriction patterns, and 476 fusion joint points were sequenced, resulting in the identification of >100 unique genes, of which >50% are unknown; i.e., they have no significant homology with sequences in the DNA data base or encode open reading frames (ORFs) with no assigned function. A map position on the *Salmonella* chromosome or virulence plasmid (pSLT) was assigned to each unique fusion by Mu*d*-P22 mapping (12). Each unique fusion was reintroduced into the wild-type chromosome and shown to map at the expected location. In the case of *ivi* fusions to known genes, all were shown to integrate at their known position in the *E. coli* or *Salmonella* genomic maps. All fusions listed in Table 1 are in the known coding sequence or in the predicted ORF of the gene indicated, with the exception of *cfa*, where the joint point is 23 bp before the ATG start codon, *cirA*, where the fusion joint point is 104 bp after the translational stop codon, and *iviXV*, where the coding sequence has not been identified.

Induction of *ivi* Fusions Is Required for Survival in the Animal under Conditions of the IVET Selection. Induction of a given *ivi* gene in the animal was assessed by the ability to synthesize sufficient quantities of the *purA* or *cat* gene products to allow sufficient bacterial growth to cause morbidity or mortality. BALB/c mice were infected with either an *ivi* or a preselected Lac^- or Lac^+ fusion strain. After 5 days of incubation, the number of bacterial cells present in the spleen was determined. β-Galactosidase assays of the Lac^- *ivi* fusions and the preselected Lac^- fusions showed similar levels of *in vitro* promoter activity when grown in LB liquid culture (data not shown). Fig. 2 shows that after i.g. infection with 10^6 cells, pIVET1 *ivi* fusion strains, MT1466 (*phoP*) and MT1733 (*iviXII*), and a preselected Lac^+ fusion strain, MT1734, exhibited a 10^6- to 10^8-fold growth advantage over two preselected Lac^- fusion strains (MT1788 and MT1735). Moreover, i.p. infection with 5×10^2 cells of either pIVET1 (MT1466 and MT1733) or pIVET8 fusions MT1461 (*iviVI-A*) and MT1501 (*iviXII*) showed a 10^4- to 10^6-fold growth advantage over the preselected Lac^- control strains. These data indicate that the induction of an individual IVET-selected fusion is required for survival in the animal under conditions of the IVET selection.

LD_{50} Studies of *ivi* Genes. *ivi* genes or operons already known to be essential for virulence were not tested here (e.g., *phoPQ*,

Table 1. *S. typhimurium* genes that are induced *in vivo*

Strain	Gene*	Function	Role in pathogenesis	Parameters†
		Regulatory genes		
MT1466	*phoP*	Virulence regulator	Invasion/macrophage survival	1BC
MT1731	*pmrB*	Polymyxin resistance	Neutrophil survival	8D
MT1396	*cadC*	Cadaverine synthesis	Acid tolerance	1A, C
MT1398	*iviXIII*‡	ChvD-like	Regulator induction	1B; 99 min
MT1632/1633	*vacB*‡/*vacC*	RNA processing	Post-transcriptional regulation	1BC/1A; 95 min/9 min
		RpoS-regulated genes		
MT1483	*spvB*	Plasmid virulence	Systemic survival	8C
MT1397	*cfa*	Membrane modification	Stationary-phase survival	1AC
MT1459	*otsA*	Trehalose synthesis	Stationary-phase/ osmoprotectant	1A
		Metabolic functions		
MT1562	*recD*	Recombination/repair	Macrophage survival	8D
MT1426	*hemA*	Catalase cofactor	Peroxide resistance	1C
MT1505	*entF*	Enterobactin synthesis	Iron acquisition	8D
MT1415	*fhuA*	Iron transport	Iron uptake	1A
MT1399	*cirA*	Colicin I receptor	Catechol transport	8D
MT1442/1443	*mgtA*‡/*mgtB*‡	Mg^{2+} transport	Mg^{2+} uptake	8CD/8C
MT1498	*iviX*‡	Heavy metal transport	Cu^{2+} homeostasis	8D; 11 min
MT1446	*ndk*	Nucleotide balance	Alarmone synthesis	1AC
		Systemic adhesin- and invasin-like genes		
MT1461	*iviVI-A*‡	Tia/Hra1-like	Adhesion/invasion	8C; 7 min
MT1461	*iviVI-B*‡	PfEMP1-like	Adhesion/invasion	8C; 7 min
		Mouse spleen and cultured macrophages		
MT1799	*iviXI*‡	Unknown	Macrophage survival	8CD; 53 min
MT1501/1733	*iviXII*	Unknown	Macrophage survival	8D/1C; 9 min
MT1500	*iviXV*	Unknown	Macrophage survival	8CD; 9 min

Listed are functions or attributes of *ivi* genes and their known or inferred roles in pathogenesis.

**ivi* fusions were cloned using a transductional method (11), and the first 200–400 base pairs were sequenced using an oligonucleotide primer that directs synthesis from the 5′ end of the selected promoterless gene (*purA* or *cat*) into the cloned fragment. Nucleotide and deduced amino acid sequences were compared with known data bases by using the FASTA and BLAST programs of GCG (Madison, WI). All fusions listed are in the known coding sequence or in the predicted ORF of the gene indicated with the following exceptions: *cfa*, where the joint point is 23 bp before the ATG start codon, *cirA*, where the fusion joint point is 104 bp after the translational stop codon, and *iviXV*, where the coding sequence has not been identified although it has been recovered from three independent experiments (from the spleen after an i.p. infection and from two cultured macrophage selections). A ‡ designation after the gene indicates that an insertion mutation in the coding sequence was isolated and assayed for a virulence defect in an LD_{50} study, 500-fold and >100-fold above the i.g. and i.p. LD_{50}, respectively. None of these mutations conferred a virulence defect by this assay.

†The numbers refer to the IVET vector used in the selection: 1 = pIVET1 (*purA*); 8 = pIVET8 (*cat*). The capital letters denote the route of delivery and the host tissue (BALB/c mice) from which the bacteria were recovered. A = i.g., small intestine; B = i.g., spleen; C = i.p., spleen; and D = cultured RAW 264.7 macrophages. Map positions in minutes are provided for *ivi* genes that have not been previously described in *Salmonella*. Map position on the *Salmonella* chromosome was determined by Mud-P22 transductional mapping (12).

spvB, *hemA*, *recBCD*). Insertion mutations (Tn*10d*-Tc and/or pGP704) in many of the *ivi* genes have been constructed and tested for virulence defects as assessed by 50% lethal dose (LD_{50}) studies. Table 1 shows the *ivi* genes tested (indicated by ‡). None of the insertions conferred a virulence defect when BALB/c mice were challenged either i.g. or i.p. at a dose 500-fold or >100-fold, respectively, above the LD_{50}. These studies show that although these *ivi* genes are not essential in these inocula to cause lethality, their induction in host tissues may reflect their contribution and perhaps requirement for growth at specific sites (see *Discussion*).

Regulatory Genes. Table 1 shows that several known regulatory genes are induced during infection, including *phoP*, *pmrB*, and *cadC*. The *phoPQ* operon encodes a two-component regulator of *Salmonella* virulence that controls the expression of functions required for invasion of mammalian cells, survival in macrophages, and resistance to low pH and to defensins (reviewed in ref. 13). Fig. 2 shows that the induction of *phoP* (MT1466) after either an i.g. or an i.p. infection is sufficient to satisfy both the mucosal and systemic *purA* requirements in the animal, which is consistent with a role at early and late stages of infection. Table 2 shows that *phoP* is autoregulated as shown previously (16) and PhoPQ controls the expression of the *ivi* operon, *pmrAB*, encoding a two-component regulatory system involved in resistance to *c*ationic *a*ntibacterial *p*roteins (CAP) of human neutrophils (17). The expression of virulence factors by such a regulatory cascade provides a mechanism to modulate the protective response.

The ability to respond to low-pH environments is an important aspect of *Salmonella* pathogenicity. *cadC* controls the expression of lysine decarboxylase, which may increase the pH of host cell organelles by the production of cadaverine, a primary amine (18). Moreover, CadC is topologically similar to ToxR, the global regulator of virulence in *Vibrio cholerae* (18, 19). Both CadC and ToxR respond to low pH and media composition, but it is unclear whether ToxR regulates polyamine synthesis in *V. cholerae* or whether CadC regulates other virulence genes in *Salmonella* spp.

Some virulence functions are utilized by both plant and animal pathogens. The *chvD* gene product of the plant pathogen *Agrobacterium tumefaciens* is involved in the expression of the VirA/VirG two- component regulatory system required for full virulence. Under conditions of low pH and phosphate starvation, ChvD is required for the induction of *virG* transcription (20). The *ivi* gene *iviXIII* is the *Salmonella* homologue of *E. coli* ORF *orf579*, encoding a predicted peptide that is ≈55% identical to the entire predicted internal fragment of ChvD. The *in vivo* induction of IviXIII in *S. typhimurium* suggests that this protein may function as a sensory element, shared by animal and plant pathogens, which modulates the expression of regulatory systems.

Two additional loci previously undescribed in *Salmonella*, homologues of *Shigella* spp. *vacB* and *vacC*, have been identified by the IVET selection. These *v*irulence-*a*ssociated *c*hromosomal loci affect virulence plasmid gene expression in *Shigella* spp. and enteroinvasive *E. coli* (EIEC). In *Shigella* spp., both VacB and

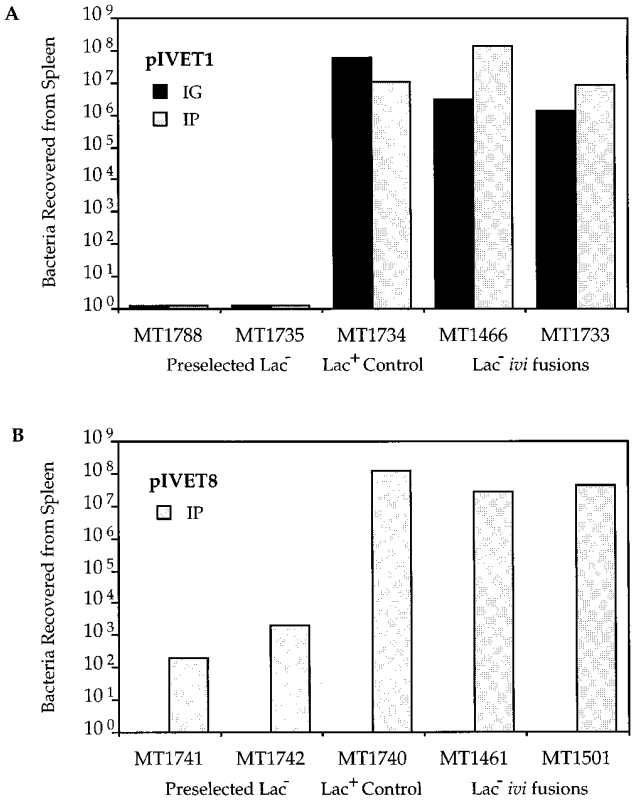

FIG. 2. Induction of *ivi* genes is required for survival in the animal under conditions of the IVET selection. BALB/c mice were infected i.g. (10^6 cells) or i.p. (500 cells) with either an *ivi* or preselected Lac$^-$ or Lac$^+$ bacterial fusion strain. The number of bacterial cells recovered from the spleen was determined after morbidity was observed in the Lac$^+$-infected controls (5 days). (*A*) pIVET1 (*purA*) i.g. and i.p. selections. (*B*) pIVET8 (*cat*) i.p. selection. The number of bacteria recovered from the spleen after i.g. (IG) or i.p. (IP) infection is indicated by dark or gray bars, respectively. The *ivi* fusion joint points are as follows: *phoP* (MT1466); *iviXII* (MT1733); *iviVI-A*; MT1461, and *iviXII* (MT1501).

VacC are involved in the post-transcriptional regulation of *ipa* (*i*nvasion *p*lasmid *a*ntigens) and *ics* (*i*ntercellular *s*pread) gene products, which are required for invasion and lateral spread within host cells (21, 22).

RpoS-Regulated Genes. The stationary-phase sigma factor, RpoS, which is required for full virulence (23), regulates the expression of at least three *ivi* fusions—e.g., *spvB* (24), which encodes a S*almonella p*lasmid *v*irulence function that facilitates growth at systemic sites of infection (reviewed in ref. 25). Table 2 shows that *spvB* is also regulated by PhoPQ, demonstrating that the expression of *spvB* is influenced by both a sigma factor and a two-component regulator. Members of the *spv* operon have also been recovered by other methods to identify genes required *in vivo* (26).

Table 2. PhoPQ regulation of *ivi* genes

ivi-lac fusion*	β-Galactosidase, units†		
	phoP$^+$ *Q*$^+$	*phoP102*::Tn*10d*-Cm	*phoQ24*
phoP	110	11	132
spvB	118	14	294
pmrB	122	31	187
mgtB	48	9	35
iviVI-A	236	5	563
ndk	18	21	21

*Wild-type, PhoP$^-$ [*phoP102*::Tn*10d*-Cm (7)], and PhoQ^c [*phoQ24*, which constitutively activates PhoP-activated genes (8)] strains were grown in Mops media (15), where Mops [3-(*N*-morpholino)propanesulfonic acid] was replaced with Mes [2-(*N*-morpholino)ethanesulfonic acid] and buffered to pH 5.5 (50 μM Mg^{2+}). The defects observed in *phoP102*::Tn*10d*-Cm may also be attributed to the lack of *phoQ*.

†Numbers given indicate β-galactosidase activities assayed according to Slauch and Silhavy (14). Units are given as (units per OD_{600} unit × ml of cell suspension) × 10^3, where 1 unit = 1 μmol of *o*-nitrophenol formed per min (n = 3, SD < 10%).

rpoS controls the expression of two other *ivi* fusions, *cfa* (*c*yclopropane *f*atty *a*cid synthase) (27) and *otsA* (*o*smoregulatory *t*rehalose *s*ynthetase) (28). Cfa introduces a cyclopropane ring into bacterial membrane fatty acids during growth under conditions of amino acid limitation, preventing the loss of cellular proteins (29). Moreover, *Mycobacterium tuberculosis cma1* encodes a similar modification that has been implicated in resistance to peroxides (30). OtsBA synthesizes trehalose, which is important for survival during osmotic (31) and thermal (28) stress. Induction of these RpoS-regulated genes suggests that nutrient limitation, osmolarity, and temperature are relevant components of the host ecology that signal the expression of virulence genes.

Metabolic Functions. The multisubunit complex RecBCD, the major recombination and repair system in bacteria, has also been implicated in superoxide resistance (32). HemA, involved in the synthesis of heme, an iron-containing compound that serves a role in electron transport processes (33), may, in part, mediate protection from oxidative damage by the synthesis of the heme component of catalase (34), although catalase mutants do not show a reduction in virulence (35). *recBD* and *hemA* are in *ivi* operons required for full virulence (32, 33), and their *in vivo* induction in the macrophage or spleen provides an added benefit to the pathogen: a protective response against protein and DNA damage resulting from the macrophage oxidative burst.

The availability of metal ions, including Fe^{2+}, Mg^{2+}, and Cu^{2+}, affects the expression of genes required for their transport as well as other virulence functions. For example, Fe^{2+} is limited in the host by the action of host iron-binding proteins. Induction of *entF*, involved in the synthesis of enterobactin, as well as the siderophore transport system, *fhuA* (ferric hydroxamate uptake), and the catechol transporter, *cirA* (colicin I receptor) (reviewed in ref. 36), reflects the pathogen's attempt to overcome this well characterized barrier to infection (reviewed in ref. 37). As expected, *entF*, *fhuA*, and *cirA* fusions are induced under iron-limiting conditions *in vitro* (U.H., C.P.C., D.M.H., F. Govantes, and M.J.M., unpublished results).

Mg^{2+} levels are estimated to be low (<50 μM) in the phagosome (38) and have been shown to induce the expression not only of two high-affinity Mg^{2+} transport systems, but also *phoPQ* and genes under its control (refs. 38 and 39; U.H., C.P.C., D.M.H., F. Govantes, and M.J.M., unpublished results). Table 1 shows that *ivi* fusions to these Mg^{2+} transport genes have been recovered from BALB/c mice (*mgtA* and *mgtB*) and cultured RAW 264.7 macrophages (*mgtA*). Moreover, Table 2 shows that *mgtB* is regulated by PhoPQ as indicated previously (39). While the induction of *mgtA* and *mgtB* may reflect the pathogen's attempt to counter the inhibitory effects of low Mg^{2+} in the phagosome, it also demonstrates the role of Mg^{2+} as an environmental signal that contributes to the expression of genes required for macrophage survival (39). As expected, *mgtA* and *mgtB* fusions are induced under Mg^{2+}-limiting conditions *in vitro* (ref. 39; U.H., C.P.C., D.M.H., F. Govantes, and M.J.M., unpublished results).

iviX has been recovered from two independent selections performed in cultured macrophages infected *in vitro*. The predicted protein sequence of IviX shows significant homology (33% identity) to heavy metal transporters of many bacteria and is regulated in response to the level of Cu^{2+} in the medium (data not shown).

In addition to metals, nucleotide availability may also serve as a signal for virulence gene expression. For example, *vacB* (discussed above) is an RNase II homologue which may serve to reconstitute the depleted nucleotide pools *in vivo* by means of increased RNA turnover. Moreover, mutations in *vacB* affect the

expression of *ipa* and *ics* virulence genes of *Shigella* spp. (21, 22). *ndk* is an *ivi* gene encoding *n*ucleoside *d*iphosphate *k*inase that maintains proper nucleotide (NTP) balance in both prokaryotes and eukaryotes. Ndk is involved in the synthesis of GTP, a precursor of the alarmone ppGpp, which is known to signal nutrient-limiting conditions (40). Moreover, Ndk-dependent alterations in metabolism during stationary phase affect *Pseudomonas aeruginosa* virulence (see *Discussion*). *ndk* is closely linked to two other independently isolated *ivi* genes, *iviVIII-A* and *iviVIII-B*, *Salmonella* homologues of the previously reported *E. coli* ORFs, *orf384* and *orf337* (41). However, their biochemical functions or roles in virulence are unknown.

Systemic Induction of Adhesin- and Invasin-Like Genes. Two *ivi* genes encoding adhesin- or invasin-like proteins have been identified from a fusion recovered from the spleen after i.p. infection, suggesting a continued systemic role for adhesion and/or invasion factors. *iviVI-A* and *iviVI-B* reside in a region of exceptionally low G+C content and thus are presumed to have been acquired by horizontal transfer (42). *iviVI-A* encodes a predicted product that shows significant sequence similarity (>35% identity) over its entire length to both enterotoxigenic *E. coli* Tia, an outer membrane protein required for toxigenic to and invasion of cultured gut epithelial cells (43), and to *E. coli* Hra1, an enteric pathogen afimbrial adhesin (44). Fig. 2 shows that induction of *iviVI-A* is required for survival in the animal under the conditions of the IVET selection.

A Tn*10d*-Tc insertion in the upstream, closely linked gene *iviVI-B* shows polarity on the transcriptional activity of *iviVI-A*, indicating these two genes are in the same operon. IviVI-B shows regions of similarity (33% to 47%) to several Duffy binding-like (DBL) domains of the malarial virulence factor PfEMP1 (*Plasmodium falciparum*-infected erythrocyte membrane protein 1) (45). PfEMP1 belongs to the plasmodial family of DBL proteins involved in red blood cell (RBC) invasion and surface modifications (reviewed in ref. 46). Table 2 shows that *iviVI-A* (and presumably *iviVI-B*) is PhoPQ regulated, indicating that genes acquired by horizontal transfer have adopted the regulatory circuitry of *Salmonella* virulence genes.

Mouse Spleen and Cultured Macrophages. *iviXI, iviXII, iviXV,* and *mgtA* represent four examples of specific *ivi* fusions recovered from spleens and from cultured macrophages. *iviXI* resides in a previously reported ORF of *Salmonella* (*orf179*), *iviXII* encodes a predicted peptide with similarity (52%) to an ORF of *Haemophilus influenzae* HI1305. *iviXV* has been recovered from three independent experiments, although the coding sequence has not been identified. Fig. 2 shows that induction of *iviXII* is sufficient for survival in BALB/c mice under both the pIVET1 (*purA*) and pIVET8 (*cat*) selections. Taken together, these data indicate that fusions recovered from cultured macrophages are expressed at levels sufficient to answer the IVET selection in the animal.

DISCUSSION

We have employed IVET, a genetic system that uses the animal as a selective medium, to enrich for bacterial genes induced *in vivo*. During infection of its host, the pathogen elaborates a broad array of regulatory, metabolic, and virulence functions that contribute to pathogenicity. Inspection of the *in vivo* induction profile reveals (*i*) many *ivi* genes encode regulatory functions that serve to enhance the sensitivity and amplitude of the *in vivo* response; (*ii*) many metabolic genes have a direct role in virulence; (*iii*) some *ivi* genes encode functions that are similar to those of pathogens that span vast evolutionary distances ranging from bacterial pathogens of plants and animals to eukaryotic human parasites; and (*iv*) the host ecology provides an environmental address to which the pathogen responds with the coordinate expression of metabolic and virulence functions.

The multifactorial nature of virulence indicates that pathogenesis is not restricted to a single linear pathway from infection to mortality. Thus, since many alternative routes of spread are available, individual mutations in genes that affect the growth of a pathogen at a specific host site may not affect lethality. The contribution of these genes will be overlooked in a standard LD_{50} assay which lacks the resolution to elaborate a specific function that is available in more defined *in vitro* or *in vivo* infection models (e.g., invasion of epithelial cells, Peyer's patches, or phagocytes). A classic example is demonstrated by the fact that mutants in the well characterized *Yersinia enterocolitica* invasion gene, *inv*, do not confer a virulence defect as measured by LD_{50} studies, but they do show a dramatic defect in the ability to invade cultured epithelial cells (80-fold) and in the ability to colonize Peyer's patches (up to 10^7-fold) early after infection (47). IVET provides a means to identify essential virulence determinants as well as those elusive functions whose role may be characterized in more defined systems.

The ability to sense and respond to environmental signals is a key component to pathogenicity. Thus, several *ivi* genes encode regulatory functions, including PhoPQ, the global regulator of *Salmonella* virulence, which is shown to be induced after either an i.g. or an i.p. infection, suggesting a role at both early and late stages of infection. PhoPQ regulates its own expression as well as that of several other *ivi* genes of known and unknown function, including the regulatory gene *pmrB*. The control of one regulatory gene by another provides a means to fine-tune and/or amplify the pathogen's response to host signals encountered during infection. Signals that induce regulatory genes may be distinct from those sensed by the proteins they encode, providing a mechanism to satisfy immediate requirements and anticipate future needs. Further, the *ivi* gene *spvB* is controlled by both the sigma factor RpoS and PhoPQ, demonstrating that virulence genes respond to independent and/or overlapping regulatory signals.

The *in vivo* induction of two genes previously described in *Shigella* spp. indicates that post-transcriptional processes make a significant contribution to virulence. *vacB* is a member of the RNase II family and may exert its effect by regulating the message stability of components required for translation of *ipa* and *ics* mRNAs. *vacC* encodes a tRNA guanine transglycosylase that exerts its effect through a specific tRNA modification that leads to increased translation of the positive regulatory element *virF* (22). The involvement of tRNAs in the expression of virulence genes has been demonstrated in uropathogenic *E. coli* (48).

The induction of four independent metal transport systems reflects an aspect of host ecology wherein metal ions and other small molecules such as nucleotides serve as cofactors for enzymatic activity and also as signals that direct bacterial gene expression. For example, the limited amount of Mg^{2+} in the phagosome may signal not only the induction of Mg^{2+} transport systems but also the expression of *phoPQ* and the genes under its control (refs. 38 and 39; U.H., C.P.C., D.M.H., F. Govantes, and M.J.M., unpublished work). Moreover, the limited availability of iron is a well characterized signal for the expression of genes involved in iron acquisition and other virulence genes in several pathogens. Despite the recognized role of iron in infection, the loss of a single gene involved in iron acquisition is not always associated with a virulence defect (37). This does not, however, diminish the contribution of each individual iron-acquisition system to pathogenicity.

The importance of Cu^{2+} transport systems that maintain Cu^{2+} balance, sufficient for enzyme activity while avoiding toxic concentrations, is demonstrated by the fact that mutations which disrupt Cu^{2+} transporters lead to defects in bacterial growth (49) or to human genetic disease (50).

De novo synthesis of pyrimidines and purines has been shown to be a strict requirement for bacterial growth and persistence under the nucleotide-limiting conditions of the host (2, 26, 51, 52). Additionally, several genes involved in the synthesis and recycling of nucleotides have answered the IVET selection, including *vacB* and *ndk*. *vacB* (RNaseII homologue)-directed alterations in RNA metabolism may provide a source of nucleotides to reconstitute the depleted pools *in vivo*. Under these conditions, *vacB* may affect the stability of a specific set

of messages whose functions contribute to virulence (e.g., *ipa* and *ics* of *Shigella* spp. discussed above). *ndk* plays a direct role in the virulence of *P. aeruginosa*. In stationary phase, the activity of Ndk is modified by protease cleavage, followed by insertion into the membrane, where it produces GTP exclusively (53), possibly for the use in the synthesis of alginate (54). In *Salmonella*, the production of GTP, a precursor to the alarmone ppGpp, may be involved in signaling nutrient limitation and directing the induction of other virulence genes. Indeed, ppGpp affects the expression of *rpoS*, a stationary-phase sigma factor required for full virulence (40).

Thus components of the host ecology such as the availability of metal ions and nucleotides serve a dual role in pathogenesis: to induce metabolic functions that overcome nutritional deficiencies and to induce virulence functions required for immediate survival and spread to subsequent anatomical sites of infection.

The need for adhesins and invasins is most often discussed in the context of early infection stages (e.g., mucosal epithelium). However, the recovery from the spleen after an i.p. infection of *iviVI-A* and *iviVI-B*, encoding predicted products that resemble adherence and/or invasion factors of other pathogens, suggests a continued need for these factors at late stages of infection. IviVI-A shows homology to Tia and Hra1, adherence and invasion factors of enteric pathogenic *E. coli*. Upstream in the same operon lies *iviVI-B*, which shows regions of similarity to the conserved DBL domains of PfEMP1, an adherence factor of the eukaryotic systemic pathogen *Plasmodium falciparum*. PfEMP1 is produced by the parasite within an infected RBC and is localized to the RBC plasma membrane, where it directs adherence to vascular epithelia, preventing elimination of the infected RBC in the spleen. The function of IviVI-B in *Salmonella* is not necessarily limited to adherence, as the DBL family of proteins are also involved in invasion, antigenic variation, and possibly chemokine signaling: a member of the DBL family (DABP) binds to the RBC Duffy blood group antigen, a receptor of a family of chemokines including interleukin 8 (46).

The low G+C content of the chromosomal region in which *iviVI-AB* resides suggests that these genes have been acquired by horizontal transfer (42). The PhoPQ regulation of *iviVI-A* (and presumably *iviVI-B*) reveals that selection has favored the coordinate expression of these acquired genes with other presumed systemic *ivi* virulence and metabolic functions of *Salmonella*, including *phoP*, *spvB*, *pmrB*, and *mgtB* (Table 2). The requirement of type III secretion systems involved in systemic survival has recently been described in studies using differential hybridization methods (55) and directed mutagenesis of a region unique to salmonellae (56). Both of these type III secretion systems map to the same region of the *Salmonella* chromosome (distinct from *iviVI-AB*) and are characterized by low G+C content. These "pathogenicity islands" are believed to have been acquired by horizontal transfer. Mutations in this region confer defects in virulence after i.p. infection and in macrophage survival, suggesting they are required at systemic sites of infection. Likewise, *iviVI-AB* were recovered from the spleen after an i.p. infection, and their PhoPQ-dependent expression (Table 2) is consistent with their systemic induction (Table 1), possibly within the macrophage as in the case of other PhoPQ-activated genes [e.g., *pagC* (57)].

The independent recovery of specific *ivi* fusions from infected mice and from cultured macrophages provides complementary information regarding both infection models: it (*i*) validates both selections, (*ii*) defines the relevant host tissue, and (*iii*) provides clues to the function of *ivi* genes. The recovery of *iviXI*, *iviXII*, *iviXV*, and *mgtA* from spleens and cultured macrophages may define the relevant mammalian cell type for the expression of these four fusions and suggests that they play a role in bacterial survival within splenic phagocytes.

The ability to sense and respond to complex and overlapping signals encountered at each anatomical site is a key component to pathogenicity. This ability results in the elaboration of *ivi* regulatory, metabolic, and virulence functions that contribute to growth and persistence in host tissues. Virulence is the sum of these contributions.

We thank Matthew K. Ritter for assistance in mapping. We also thank Laszlo Csonka for critically reading the manuscript. This work was supported by National Institutes of Health Grant AI36373, American Cancer Society Junior Faculty Research Award 554, and a Beckman Young Investigator Award (M.J.M.), and by American Cancer Society Grant IRG-158 (P.C.H.).

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