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Identification of *Escherichia coli* Genes That Are Specifically Expressed in a Murine Model of Septicemic Infection

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Identification and characterization of bacterial genes that are induced during the disease process are important in understanding the molecular mechanism of disease and can be useful in designing antimicrobial drugs to control the disease. The identification of in vivo induced (*ivi*) genes of an *Escherichia coli* septicemia strain by using antibiotic-based in vivo expression technology is described. Bacterial clones resistant to chloramphenicol in vivo were recovered from the livers of infected mice. Most of the *ivi* clones were sensitive to chloramphenicol when grown in vitro. Using reverse transcription-PCR, it was demonstrated that selected *ivi* clones expressed *cat* in the livers of infected mice but not during in vitro growth. A total of 750 colonies were recovered after three successive rounds of in vivo selection, and 168 isolated *ivi* clones were sequenced. The sequence analysis revealed that 37 clones encoded hypothetical proteins found in *E. coli* K-12, whereas 10 clones contained genes that had no significant homology to DNA sequences in GenBank. Two clones were found to contain transposon-related functions. Other clones contained genes required for amino acid metabolism, anaerobic respiration, DNA repair, the heat shock response, and the cellular repressor of the SOS response. In addition, one clone contained the aerobactin biosynthesis gene *iucA*. Mutations were introduced in to seven of the identified *ivi* genes. An in vivo mouse challenge-competition assay was used to determine if the mutants were attenuated. The results suggested that these *ivi* genes were important for survival in vivo, and three of the seven mutant *ivi* clones were required for successful infection of mice.

Escherichia coli is a common gram-negative, enteric organism that also is an important etiological agent causing bloodstream infections in humans, particularly nosocomial septicemia (22, 37, 43). *E. coli* has been reported to be responsible for more than one-quarter of all septicemic infections (11). Individuals who are in a high-risk category for disease are the elderly and hospitalized patients. In the elderly, the fatality rate due to *E. coli* septicemia is 6.9% (2). *E. coli* strains expressing the K1 antigen also are important extraintestinal pathogens and are associated with neonatal meningitis. The incidence of *E. coli* K1-induced meningitis is 0.1 per 1,000 live births (7), and fatality rates range from 25 to 40% (40).

One approach to develop new treatments for *E. coli* infections is to identify and characterize genes that are expressed in vivo during disease and consequently those that are essential for the survival of bacteria in the infected host. During septicemia, *E. coli* is exposed to a unique set of growth conditions and clearance mechanisms. A number of growth conditions that affect the expression of virulence-associated *E. coli* genes, such as iron and nutrition starvation, oxygen tension, pH, and stress are well documented (29). These in vitro growth techniques mimic individual growth conditions present in the infected host but do not replicate the highly complex environment that a pathogen encounters in the infected host. In the complex host-pathogen interaction, pathogenic bacteria also must evade the host immune system in a nutrition-depleted environment. These hostile environments require that a successful pathogen must express specific sets of genes in response to the growth milieu. Most pathogenic bacteria respond to the interaction with eukaryotic cells by expressing various virulence factors, including adhesins (9, 17, 18), toxins (6), and iron uptake systems (45). In recent years, techniques that could be used to identify bacterial genes that are uniquely expressed during infection have been developed (15, 26, 27). In vivo expression technology (IVET) has been used as a positive selection tool for in vivo-induced (*ivi*) genes. This technique was originally developed for *Salmonella enterica* serovar Typhimurium and has been useful in the identification of previously uncharacterized genes (26). The original technique relied on complementation of an auxotrophy during selection in a murine infection model. Auxotrophic strains survived only if a cloned chromosomal DNA fragment possessing in vivo promoter activity promoted expression of a promoterless auxotrophic gene integrated into the bacterial chromosome. Using a similar technique, the auxotrophic marker was successfully replaced by a promoterless chloramphenicol acetyltransferase (*cat*) gene to isolate *ivi* genes of *S. enterica* serovar Typhimurium (27). In addition, other molecular techniques, such as signature-tagged mutagenesis to negatively select for tagged mutant genes required for pathogenesis (15), in vivo-induced antigen technology (IVIAT) selecting for *ivi* genes by screening expression libraries with a patient's serum after absorbing with cells of the pathogen grown in vitro (13), and a promoter trap technique that relies on genetic recombination as a reporter of gene expression (24), are being used to identify in vivo-expressed genes. These techniques have been successfully used to identify *ivi* genes of *Yersinia enterocolitica* (46), *S. enterica* serovar Typhi (38), *Pseudomonas aeruginosa* (42), *Actinobacillus*

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristic(s)	Reference or source
E. coli strains		
i484	O25:H autoaglutinating; human isolate	This study
i659	Derivative of i484; Sm^r	This study
i665	*E. coli* strain HB101 containing pKK232-8	This study
i668	*E. coli* i659 containing pKK232-8	This study
i673	In vivo-induced clone	This study
i675	In vivo-induced clone	This study
i681	In vivo-induced clone	This study
i682	In vivo-induced clone	This study
i683	In vivo-induced clone	This study
i684	In vivo-induced clone	This study
i686	In vivo-induced clone	This study
i689	In vivo-induced clone	This study
HB101	*Δ(gpt-proA)62 leuB6 thi-1 lacY1 recA rpsL20* (Str^r) *ara-14 galK2 xyl-5 mtl-1 supE44 Δ(mcrBC-hsdRMS-mrr)*	3
SM10 λpir	*thi-1 thr-1 leuB6 supE44 tonA21 lacY1 rec*::RP4-2-Tc::Mu Km^r	28
Plasmids		
pKK232-8	Amp^r Cm^s; ColE1 origin	4
pGP704	Amp^r; R6K origin	28

pleuropneumoniae (10), *Staphylococcus aureus* (24), and *Pasteurella multocida* (16).

In this study we sought to identify genes of an *E. coli* strain that causes nosocomial septicemia that were expressed exclusively during disease. To fulfill this objective, we developed a variation of the original IVET procedure. The procedure employs a nonchromosomal integration system with a low-copy-number plasmid for large-scale screening of chromosomal fragment::*cat* fusions to isolate *ivi* genes. The *ivi* genes identified included genes similar to uncharacterized open reading frames (ORFs) of *E. coli* K-12, genes that have no similarity to *E. coli* K-12, and genes that have no homologues in the public databases of DNA and protein sequences.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture media. *E. coli* strain i484 was isolated from a human with septicemia. It is sensitive to all antibiotics tested. It has the serotype O25:H, autoagglutinating. Its derivatives and other *E. coli* strains used in this study are listed in Table 1. Luria-Bertani (LB) medium was routinely used to grow *E. coli* strains either in liquid culture or on agar plates at 37°C. Final concentrations of the antibiotics in growth media were as follows: ampicillin, 100 μg/ml; chloramphenicol, 25 μg/ml; and streptomycin, 20 μg/ml. To induce iron limitation in minimal medium and LB agar plates, 2,2′-dipyridyl (ICN Biomedicals, Inc., Irvine, Calif.) was used at concentrations ranging from 25 to 150 μM.

Construction of genomic library. A genomic library of *E. coli* i484 was constructed in plasmid pKK232-8, a promoter selection vector (Pharmacia, Piscataway, N.J.). Chromosomal DNA was isolated from *E. coli* strain i484 by a procedure employing cetyltrimethylammonium bromide (1) and was partially digested with restriction endonuclease *Sau*3AI. DNA fragments of 0.5 to 2 kb were ligated into the *Bam*HI site of the vector pKK232-8. After transformation of the recombinant DNA into *E. coli* strain HB101, a total of 4×10^4 colonies were collected by plating on LB agar containing ampicillin. Random clones were picked, and the average insert size of the genomic library was shown to be approximately 1 kb. This represented an eightfold redundancy, based on the size of the *E. coli* K-12 genome. Subsequently, plasmid DNA was prepared from the recombinant strain and electroporated into the streptomycin-resistant mutant of i484, strain i659.

***ivi* gene selection in mouse septicemia model.** Six- to 8-week-old female ICR mice (Harlan Dawley, Indianapolis, Ind.) were challenged by intraperitoneal injection with cells from the genomic library. Bacterial cells in exponential growth were harvested and resuspended in phosphate-buffered saline. Based on preliminary data (see Results) the challenge dose was set at 10^8 CFU. Immediately after challenge of a group of five mice, mice were injected intraperitoneally with 500 μg of chloramphenicol. A total of five doses of antibiotic were administered at 4-h intervals. At 24 h postchallenge, mice were euthanized with CO_2. The livers were isolated, homogenized in 10 ml of sterile H_2O, plated on LB agar plates containing 20 μg of streptomycin per ml, and incubated overnight at 37°C. Bacterial colonies were then replica plated onto LB agar plates containing 100 μg of ampicillin per ml and on plates with 100 μg of ampicillin per ml and 20 μg of chloramphenicol per ml. Subsequently, plated clones that were sensitive to chloramphenicol were pooled and subjected to two more rounds of in vivo selection.

To determine the effects of mutations in the *ivi* genes of strain i484 in vivo, a mouse competition infection model was used. A mixture containing an equal number of cells of the mutant and wild-type strains was used to challenge mice. Twenty-four hours postchallenge, bacteria were recovered from the livers of challenged mice and plated on LB agar. The wild-type strain was resistant to streptomycin, whereas the mutant encoded resistance to ampicillin. Survival of each bacterial strain was scored on LB agar plates containing either ampicillin or streptomycin.

Cycle sequencing and DNA sequence analysis. Double-stranded plasmid DNA was prepared using the Qiagen (Valencia, Calif.) plasmid kit. A sequencing primer, 5′-GAAAATCTCGTCGAAGCTC-3′ (Ocat1), that read into the flanking cloned DNA fragment was designed within *cat*. DNA sequencing was performed using the *Taq* DyeDeoxy terminator cycle sequencing kit (Perkin-Elmer, Foster City, Calif.). Sequencing reactions were analyzed using an automated DNA sequencer (model ABI-373; Applied Biosystems, Foster City, Calif.). DNA and protein sequences were analyzed using the Wisconsin package version 9.1 (Genetic Computer Group, Inc., Madison, Wis.). Homology searches and sequence comparisons were performed with sequences in GenBank, EMBL, and unfinished genome databases through National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). Sequences that had no homologue in the GenBank were analyzed using GeneMark (http://dixie.biology.gatech.edu/GeneMark/gmhmm2_prok.cgi) to predict the 5′ ends of the gene (25).

Isolation of RNA and RT-PCR. RNA was isolated from the livers of infected and noninfected mice and from *E. coli* cells grown in LB medium by using the RNeasy total RNA kit (Qiagen Inc., Valencia, Calif.). To isolate RNA from mouse liver, the liver was removed, immediately frozen in liquid nitrogen, and ground to fine powder with a mortar and pestle. Approximately 50 mg of ground tissue containing 3×10^4 bacterial cells or 10^9 *E. coli* cells grown in LB medium were used to isolate total RNA. The *cat* gene transcript was then detected by reverse transcription-PCR (RT-PCR). Two primers were designed, 5′-TTCT GCCGACATGGAAGCCATCAC-3′ (RP1) and 5′-CCTATAACCAGACCGT TCAGCTGG-3′ (FP1), that were specific to *cat* and that amplified a 516-bp internal fragment of *cat* mRNA. cDNA synthesis and PCR amplification were performed using *Tth* DNA polymerase (Boehringer Mannheim, Indianapolis, Ind.). RT was performed in the presence of 0.9 mM $MnCl_2$ at 66°C for 20 min. After cDNA synthesis, the forward primer and 1.5 mM $MgCl_2$ were added to the reaction mix, followed by PCR amplification. PCR amplification was for 36 cycles as follows: denaturation at 95°C for 2 min, annealing at 60°C for 1 min, and elongation at 72°C for 1.5 min. At the end of the 36th cycle, reaction mixtures were left at 72°C for another 5.5 min. Ten microliters of reaction mixture was loaded on a 0.8% agarose gel and subjected to electrophoresis. To confirm that the amplified fragment indeed was a product derived from RNA and not the result of the amplification of the genomic copy of the *cat* gene, the mRNA preparations were subjected to degradation with DNase-free RNase. This was followed by PCR amplification.

Site-directed mutagenesis. A PCR-based method was used to introduce mutations into seven in vivo-expressed (*ivi*) genes. Using DNA sequence information on the cloned *ivi* gene, oligonucleotide primers that were specific for the 3′ end of each cloned DNA fragment were designed. The primers were designed so that frameshift mutations resulting in a premature stop codon were introduced in the *ivi* sequence, or specific stop codons were designed within the primers (Table 2). A second forward primer (5′-CACACCGCATTATGGTGCACTCT CAGTACAATCTGC-3′) that was adjacent to the cloning site in the vector pKK232-8 was designed. Prior to PCR amplification, 200 pmol of each primer was phosphorylated using 20 U of T4 polynucleotide kinase (Gibco/BRL, Grand Island, N.Y.), 1 mM ATP, and 6 μl of forward reaction buffer (350 mM Tris-HCl [pH 7.6], 50 mM $MgCl_2$, 500 mM KCl, 5 mM 2-mercaptoethanol) in a total volume of 30 μl. Reaction mixtures were incubated at 37°C for 30 min, followed by heat inactivation of T4 polynucleotide kinase at 65°C for 15 min. The *ivi* gene fragments were then amplified by PCR using *Pwo* DNA polymerase (Boehringer Mannheim). Amplification by *Pwo* DNA polymerase results in blunt-ended amplified fragments. The conditions for PCR included one cycle of denaturation at 95°C for 2 min followed by 30 cycles of denaturation at 95°C for 15 s, annealing

TABLE 2. Primers used for site-directed mutagenesis

Clone	Gene or ORF	Mutagenic primer
Δivi814	*bglG*	GCCGCAAAGCGGTAGATCTGGCAAGTTATGACGGAG
Wild type	*bglG*	GCCGCAAAGCGGTAGAGGGCAAGTTATGACGGAG
Δivi814	*o761*	GGATCAAGTTATCTAGAAAAAGGCGTGTTTTCAAGCCAGCAATAACGAAATAATTGCCG
Wild type	*o761*	GGATCAAGTTATCTGAAAAAGGCGTGTTTCTCGCCAGCAATAACGAAATAATTGCCG
Δivi815	*ycjF*	GCAAAAGCAATATTCAGCAAGGTACCAGCTTAAACAGGC
Wild type	*ycjF*	GCAAAAGCAATATTCAGCAATACCAGCTTAAACAGGC
Δivi816	*clpB*	CCCGGATAAATTCACGATCGCGTTTAATAAAAGAATGGATCC
Wild type	*clpB*	CCCGGATAAATTCACGTTCGCGAATAAAAGAATGGATCC
Δivi817	—[a]	GCCGTAGCGATTTAAAGATGCAATAACGGCG
Wild type	—	GCCGTAGCGATTTAGATGCAATAACGGCG
Δivi818	ORF V	CATCACCAGGATGGCTTTAAAGCAATAAGAGTTGGCC
Wild type	ORF V	CATCACCAGGATGGCTTTGAGCAATAAGAGTTGGCC
Δivi819	*aroA*	CAGTCTTATCAGTCCCCGGGTAGCTATTTGG
Wild type	*aroA*	CAGTCTTATCAGTCTCCGGGTACTTATTTTGG

[a] —, no similarity to sequences in GenBank.

at 60°C for 30 s, and polymerization at 72°C for 1 min during the first 10 cycles, for 1 min 20 s during the following 10 cycles, and for 1 min 40 s for the next 5 cycles, with a 2-min elongation time for the last 5 cycles. At the end of the reaction, the mixture was incubated at 72°C for 5 min. The amplified DNA fragments were ligated into the *Sma*I site of suicide vector pGP704 (30), and the mutations were introduced into i484 as described previously (12). Suicide vector pGP704 contains the RP4 *mob* site and R6K origin of replication, which requires the π protein to function. The π protein is provided in *trans* in the vector-containing strain by λpir. The recombinant suicide vector was electroporated into *E. coli* strain SM10 λpir. Conjugation was carried out on LB agar plates between recipient strain i659 and donor strain *E. coli* SM10 λpir carrying recombinant pGP704. Transconjugants were selected for resistance to ampicillin and streptomycin. The isolated transconjugants were due to the integration of the recombinant pGP704 by homologous recombination between the target *ivi* gene on the chromosome and the mutated copy of the gene on recombinant pGP704. The recombination event resulted in production of a truncated target *ivi* gene and a second mutated copy of the *ivi* gene. Chromosomal integration was confirmed by Southern blot hybridization using *Sma*I-linearized wild-type plasmid pGP704 as a probe labeled with ^{32}P.

RESULTS

Selection of *ivi* genes in a mouse model of septicemia. The experimental approach to identify bacterial genes that were specifically induced during *E. coli* septicemia employed a promoter detection cloning vector and an in vivo antibiotic selection mouse infection model. *E. coli* strain i484, which is a clinical isolate from a patient with septicemia, was used as the infectious agent. A genomic library was created in the plasmid pKK232-8 (4). pKK232-8 contains a promoterless chloramphenicol acetyltransferase (*cat*) gene, which was used as a gene reporter. Prior to in vivo selection, challenge doses and antibiotic treatment conditions were selected to optimize the selection process. First it was necessary to select a challenge dose that would result in disease but not in rapid death of the challenged mice. An intraperitoneal challenge dose that resulted in survival of all mice at 24 h postchallenge and in death by 48 to 72 h was sought. Doses of 10^7, 10^8, 10^9, and 10^{10} CFU were used to challenge mice. At the lowest challenge dose, no significant sign of disease was observed within 72 h. In addition, bacteria were not recovered from the livers of these animals. In contrast, the higher doses (10^9 and 10^{10} CFU) resulted in severe disease within an hour of intraperitoneal challenge and caused death of all of the challenged mice within 6 h postchallenge. With a challenge dose of 10^8 CFU per mouse, signs of disease appeared within 5 h postchallenge but all mice remained alive at 24 h postchallenge; 50% of the mice died in 48 h. This dosage was used for all subsequent challenge experiments.

Since the selection model being developed sought to identify clones that became resistant to chloramphenicol in vivo, it was necessary to determine a chloramphenicol dosing that would protect mice after challenge with chloramphenicol-sensitive *E. coli*. Mice were challenged intraperitoneally with i484 (2×10^8 CFU) and were given intraperitoneal injections of 0.5 mg of chloramphenicol per mouse at the time of challenge and every 4 h. After five doses of chloramphenicol, the *E. coli* strain was completely cleared from the livers of challenged mice. This antibiotic treatment protocol was used in the subsequent in vivo selection challenges.

As described in Materials and Methods, a genomic library of *E. coli* i484 was created in the vector pKK232-8. The average insert size was 1 kb. DNA fragments from *E. coli* i484 possessing functional promoters were expected to promote resistance to chloramphenicol. The library was screened in vitro prior to passage through the mouse selection model for sensitivity to chloramphenicol. Approximately 96% of the clones contained inserts that did not express the reporter gene for chloramphenicol acetyltransferase and thus were sensitive to chloramphenicol. The library was used to challenge mice that were subsequently treated with chloramphenicol to select for clones expressing the reporter gene in the infected host. It was reasoned that treatment with chloramphenicol would enrich for library clones that contained promoters that were active in vivo. The mice were euthanized at 24 h postchallenge, and 1.6×10^4 *E. coli* colonies were recovered from mouse liver. In vitro growth on plates containing either streptomycin or streptomycin plus chloramphenicol showed that 18% were resistant to chloramphenicol. This represented a 4.5-fold increase of in vitro chloramphenicol-resistant colonies. Colonies sensitive to chloramphenicol in vitro were presumed to contain genes expressed in vivo only or those that escaped selection by chloramphenicol. The chloramphenicol-sensitive colonies were pooled and subjected to a second round of screening. Pooled bacterial colonies were used to infect mice, followed by the chloramphenicol treatment. After the second round of enrichment, 99.96% of the colonies were sensitive to chloramphenicol in vitro. The in vitro chloramphenicol-sensitive colonies

were subjected to a third round of in vivo selection under the same conditions, and a total of 750 bacterial colonies were recovered. All of the isolated colonies after final selection were sensitive in vitro to chloramphenicol (25 μg/ml).

Demonstration of in vivo specific induction of *ivi* clones. Seven hundred fifty of the *ivi* colonies were obtained after three cycles of challenge in chloramphenicol-treated mice. To confirm that the chloramphenicol-treated mouse model indeed selected for clones that expressed *cat* in vivo, the bacterial cells recovered after the third round of *ivi* selection were pooled and used to challenge a group of chloramphenicol-treated mice. A second group of chloramphenicol-treated mice were challenged with the chloramphenicol-sensitive parental *E. coli* strain i484 containing the plasmid pKK232-8. After 24 h postchallenge, the parental strain was completely cleared from mouse livers, whereas 6×10^6 bacterial cells were recovered from the livers of mice challenged with the mixture of *ivi* clones. These results are consistent with the conclusion that promoters were present upstream of *cat* in some of the clones that were active during growth in mouse liver. It was considered possible that some or all of the clones simply represented cells that grew poorly or not at all in the liver and therefore were transiently resistant to chloramphenicol. To confirm that the apparent resistance to chloramphenicol in vivo was due to expression of the reporter gene for chloramphenicol acetyltransferase, an RT-PCR method was used to detect the presence of *cat*-specific transcripts. RNA was prepared from the livers of mice challenged with different *ivi* clones. RNA also was obtained from cells grown in vitro. In addition, total RNA was isolated from in vitro-grown *E. coli* strain i484 containing pKK232-8, from liver from a mouse challenged with *E. coli* strain i484, and from uninfected mouse liver. Using these RNA preparations, RT-PCR amplification was performed using primers that amplified a 516-bp fragment of *cat*. The 516-bp fragment was detected only from mouse livers infected with *ivi* clones (Fig. 1). All other RNA preparations, including in vitro-grown *ivi* clones, did not yield any amplification of this DNA fragment, confirming that the isolated *ivi* genes were exclusively induced during the process of disease in host tissues and not during in vitro growth. Subsequently, RNA preparations were subjected to digestion with DNase-free RNase followed by PCR amplification to verify that the amplification product was due to RNA and not a genomic copy of *cat*. No PCR amplification was observed when RNA was digested with RNase, demonstrating that amplification of the 516-bp fragment by *ivi* clones grown in mice was the result of reverse transcription and amplification of *cat*-specific mRNA (Fig. 1b). When DNase was used to digest any DNA in the RNA preparations, the 516-bp fragment was still produced by the *ivi* clones grown in vivo. These results further confirm the conclusion that isolated *ivi* clones were exclusively expressed in infected host tissues and not during in vitro growth in LB broth.

In vitro analysis of *ivi* clones. Although the 750 isolated *ivi* colonies were sensitive to 25 μg of chloramphenicol per ml in vitro, it was speculated that the isolated *ivi* genes may vary in promoter strength. Therefore, the MICs of chloramphenicol may not be the same for all of the isolated *ivi* clones when they are grown in vitro. The MIC for *E. coli* strain i484 containing pKK232-8 is 5 μg/ml. To determine if basal levels of the promoter::*cat* fusions might vary, the *ivi* clones were plated on

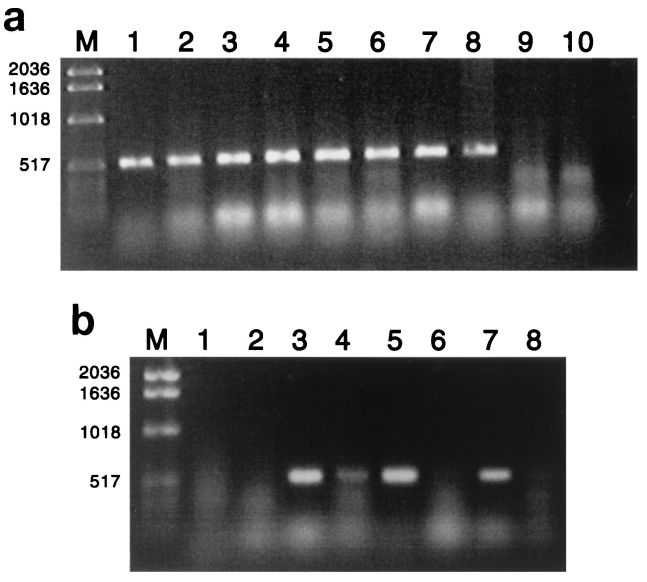

FIG. 1. (a) RT-PCR was performed to detect the presence of *cat* mRNA in *ivi* strains i673, i675, i681, i682, i683, i684, i686, and i689 (lanes 1 to 8, respectively); RNA was isolated from infected mice livers. Lane 9, RNA isolated from the liver of a mouse challenged with the wild-type *E. coli* strain i484 carrying pKK232-8; lane 10, RNA from the liver of an uninfected mouse; lane M, molecular size marker (numbers indicate base pairs). (b) RT-PCR was performed with RNA from in vitro-grown *E. coli* strain i484 containing pKK232-8 (lane 1), RNA from in vitro-grown *ivi* clones (lane 2), and RNA from the livers of mice challenged with *ivi* clones i673, i681, and i689 (lanes 3, 5, and 7, respectively). In addition, the RNAs from the three *ivi* clones were treated with RNase prior to RT-PCR (lanes 4, 6, and 8). Lane M, molecular size marker.

LB agar plates containing 5, 10, 15, 20, or 25 μg of chloramphenicol per ml. While all of the clones were sensitive to chloramphenicol at 25 μg/ml, 6% of the clones were resistant to chloramphenicol at 20 μg/ml. The number of resistant clones increased to 9, 18, and 34% when the concentration of chloramphenicol was 15, 10, or 5 μg/ml, respectively. Thus, some of the clones contained promoters that were expressed at low levels in vitro.

We expected that some of the *ivi* clones would be iron regulated. Therefore, the *ivi* clones were grown on minimal medium containing various concentration (25 to 150 μM) of 2,2′-dipyridyl and 25 μg of chloramphenicol per ml. Two clones that expressed *cat* during iron-limiting growth were identified. The remaining colonies (748) remained sensitive to chloramphenicol when subjected to these growth conditions and likely did not contain promoter::*cat* fusions that were controlled by iron limitation.

Sequence analysis of *ivi* clones. A total of 168 *ivi* cloned inserts were sequenced, and the nucleotide sequences and predicted amino acid sequences were analyzed. Of the 168 clones that were sequenced, 40 were found once, 37 were found twice, and 18 were found three times, for a total of 95 unique sequences. Sequence analysis revealed that 53 isolated *ivi* clones were previously characterized genes of *E. coli* K-12 (Table 3). Eighteen of the isolated *ivi* clones contained genes of *E. coli* K-12 that had been assigned putative functions based on sequence similarities to other known genes. In addition, 14 *ivi*

TABLE 3. List of *E. coli* genes identified to be in vivo induced

Class	Gene or ORF	Map position	Product or function
Amino acid metabolism	*dadX*	26.688	Alanine racemase
	aroA	20.651	Chorismate biosynthesis
	argD	75.155	Acetylomithine aminotransferase
	metK	66.492	*S*-Adenosylmethionine synthetase
RNA and protein biosynthesis, degradation, and modification	*me*	24.582	mRNA turnover, maturation of 5S RNA
	rrsB	89.762	16S rRNA
	tufB	89.962	Elongation factor Tu
	fdhA	80.994	Selenocysteine synthesis
	msrA	95.687	Peptide methionine sulfoxide reductase
	hslV	88.794	Heat shock protein VU, proteasome-related peptidease subunit
	clpA	19.885	ATP-binding component of serine protease
	prpB	7.499	Phosphoprotein phosphatase
DNA replication, repair, restriction, and modification	*dinD*	82.242	DNA damage-inducible protein
	dinG	17.940	Probably ATP-dependent helicase
	parE	68.363	DNA topoisomerase IV subunit B
	uvrD	86.126	DNA-dependent ATPase I and helicase II
	uvrB	17.519	DNA repair; excision nuclease subunit B.
	dnaA	83.634	DNA biosynthesis, initiation of chromosome replication, can be transcription regulator
	recG	82.403	DNA helicase, resolution of Holliday junction, branch migration
	IS*911*	97.134	New member of the IS*3* group of insertion sequences
	trs5	5.891	Transposon-related function
Cell division	*ftsX*	77.570	Cell division membrane protein
Carbohydrate metabolism	*pgm*	15.364	Phosphoglucomutase
	bgl	83.8	β-Glucoside metabolism
	malQ	76.427	Maltose degrading enzyme/amylomaltase
Surface structure	*csgA*	23.79	Fibronectin and Congo red binding curli pili of *E. coli*
Peptidoglycan biosynthesis and metabolizing enzymes	*slt*	99.764	Soluble lytic murein transglycosylase
	bacA	68.998	Bactracin resistance, possibly phosphorylates undecaprenol
Transport system	*ugpB*	77.354	*sn*-Glycerol 3-phosphate transport system, periplasmic binding protein
	fepE	13.310	Ferric enterobactin (enterochelin) transport
	iucA		Aerobactin biosynthesis
	dcuA	94.047	Membrane transport of aspartase
	trkH	86.884	Potassium uptake.
	gltS	82.451	Sodium/glutamate symport carrier protein
Outer membrane proteins	*nlpA*	82.704	Lipoprotein-28
	pldA	86.275	Outer membrane phospholipase A
Anaerobic respiration	*narW*	33.08	Appears to be essintial for nitrate reductase activity, probably by promoting the correct association of the alpha and beta subunits
	hybE	67.648	Energy metabolism, carbon: anaerobic respiration.
Drug or analog sensitivity Macromolecule biosynthesis and degradation	*tehA*	32.303	Tellurite resistance
	menG	88.731	Menaquinone biosynthesis protein
	menA	88.743	Menaquinone biosynthesis protein
	birA	89.9	Biotin-[acetyl-coenzyme A-carboxylase] ligase
	fadB	86.79	Fatty acid oxidation pathway
	tesB	10.207	Fatty acid and phosphatidic acid biosynthesis
	gloA	37.202	Methylglyoxal metabolism
	pntB	36.062	Beta subunit of pyridine nucleotide transhydrogenase
	appA	22.414	Phosphoanhydride phosphorylase
	mog	0.200	Required for efficient incorporation of molybdate into molybdoproteins
	udk	46.136	Uridine kinase
Energy metabolism	*aceE*	2.651	Pyruvate dehydrogenase
	glxB2	11.575	Glyoxylate-induced protein
	acn	28.752	Aconitate hydratase
Cellular repressor of the SOS response	*dinR*		Cellular repressor of the SOS response in *Bacillus subtilis*
Unclassified	*yejF*	49.024	Putative ATP-binding component of a transport system
	yehU	47.658	Putative 2 component sensor protein
	hslU	88.765	Putative heat shock protein HslVU, ATPase-subunit, homologous to chaperones
	yihK	87.428	Putative GTP-binding factor
	b1284	28.919	Putative DEOR-type transcriptional regulator
	o169	9.57	Putative regulator of flagellar biosynthesis, transcription initiation factor?
	o808	35.70	Putative oxidoreductase
	f344	39.40	Putative DNA helicase II
	o196	95.651	Putative transport protein
	o275	8.32	Probable ABC transporter permease protein
	f278	21.41	Probable ABC transporter permease protein
	o251	41.83	Hypothetical ABC transporter
	o127	90.77	Putative arginine biosynthesis
	f216	23.749	Putative regulator

Continued on next page

TABLE 3—*Continued*

Class	Gene or ORF	Map position	Product or function
	hycF	61.265	Probable iron-sulfur protein of hydrogenase 3
	b1806	40.7	Putative outer membrane protein
	yhjE	79.16	Putative transport protein
	f290	87.667	Putative aldose-1-epimerase
Unknown function	*ygaF*	60.095	Hypothetical protein
	pfs	3.846	Hypothetical protein
	yddE	33.912	Hypothetical protein
	yfhH	58.123	Hypothetical protein
	ycaJ	20.202	Hypothetical protein encoded in serS 5′ region
	f178	38.94	Hypothetical protein
	375	87.043	Hypothetical RNA methyltransferase
	o425	87.551	Hypothetical protein
	ycjF	29.823	Hypothetical protein
	o423	78.000	Hypothetical protein
	b2434	54.96	Hypothetical protein
	b2362	53.3	Hypothetical protein
	b1839	41.4	Hypothetical protein
	o416	85.66	Hypothetical protein encoded in rffE-rffT intergenic region

clones were hypothetical ORFs of *E. coli* with no assigned functions. Among the isolated *ivi* genes, 10 clones were unique in that they had no homologue in GenBank and databases of unfinished genomes; these sequences were submitted to GenBank (Table 4). These clones were analyzed using GeneMark version 2.4, a gene identification and ribosomal binding site prediction program. Shine-Dalgarno-like sequences were identified in eight of the unique *ivi* clones, followed by an initiation codon. The putative ribosomal binding sites were in the correct orientation to express the reporter *cat* gene. Putative ribosomal binding sites were not identified in two of the unique *ivi* clones.

In vivo analysis of *ivi* mutants. Since the *ivi* genes of *E. coli* strain i484 were induced exclusively during infection of mice, they may encode factors required for survival in the infected host. Therefore, mutations in such genes could affect the survival of the clones in the hostile environment of the host. We examined this hypothesis by introducing mutations by site-directed mutagenesis in seven selected *ivi* genes. These mutants were then used in the competitive infection model. For this model, a mutant was mixed with an equal number (10^8) of wild-type i659 cells and used to challenge a group of 10 mice. Chloramphenicol selection was not used. At 24 h postchallenge, the mice were euthanized and the concentrations of the mutant and the parent were determined by plating on separate antibiotic-containing plates (streptomycin for the parent and ampicillin for the mutant). The *ivi* genes selected for mutagenesis were a cloned gene with no significant similarity to known sequences, an ORF of unknown function (*o761*), *ycjF* (a gene that encodes a hypothetical protein), one gene with similarity to serine/threonine protein phosphatase (ORF V), and three genes of known function (*aroA*, *bglG*, and *clpB*). The results of the challenges are shown in Table 5. With the exception of *clpB* (Δivi816) and *aroA* (Δivi819), all mutants tested were attenuated.

DISCUSSION

During septicemic infections, bacterial pathogens such as *E. coli* encounter unique environments to which the pathogen responds by the coordinated expression of regulatory, metabolic, and virulence genes (9, 29). Expression of these genes in the infected host tissues contributes to the survival and successful colonization of these habitats, which otherwise would be too hostile for commensal organisms. Identification of these uniquely expressed genes is important both in understanding the infection process and for the development of new approaches to control the pathogen. For example, the identification of some genes could lead to novel discoveries of new antibiotics. During the last few years, various techniques that have been used to identify bacterial genes that are induced only during the infection process have been reported (13, 15, 24, 27). IVET, which was described by Mahan et al. (26), was developed by using an auxotrophic complementation scheme. In its original form, IVET employed an *S. enterica* serovar Typhimurium *purA* mutant to select for *ivi* genes. In this technique, the promoterless auxotrophic gene *purA* was fused with a reporter *lacZ* gene. DNA fragments from a pathogenic test strain were inserted in front of *purA*, followed by homologous recombination into the genome of an *S. enterica* serovar Typhimurium *purA* deletion mutant. These cells were then used to challenge mice. If a promoter was in the cloned DNA fragment and if the promoter was active in vivo, it would drive the expression of *purA* and consequently complement the auxotrophy. Since *purA* mutants are avirulent, only those clones expressing *purA* are viable in vivo. The *lacZ* reporter was then

TABLE 4. In vivo-induced *E. coli* genes submitted to GenBank

Clone	Accession no.
ivi931	AF249904
ivi932	AZ877598
ivi933	AZ877599
ivi934	AF249901
ivi935	AZ877597
ivi936	AZ877600
ivi937	AF249903
ivi938	AF249902
ivi939	AZ877601
ivi940	AZ877602

TABLE 5. Comparisons of mutant and wild-type growth in the mouse competition model of septicemia

Mutant	Gene or ORF	Function	Competition index (CFU of wild type/ CFU of mutant)
Δivi813	*bglG*	β-Glucoside-metabolizing enzyme	2.1[a]
Δivi814	*o761*	Unknown	1.8[a]
Δivi815	*ycjF*	Hypothetical protein	81.4[a]
Δivi816	*clpB*	ATP-dependent protease subunit	2.7
Δivi817		No significant similarity to sequences in GenBank	28.5[a]
Δivi818	ORF V	Similar to serine/threonine protein phosphatase	7.6[a]
Δivi819	*aroA*	Aromatic amino acid biosynthesis	2.7

[a] Significant ($P > 0.05$) based on Wilcoxon signed rank test.

used to measure gene expression. With IVET, *ivi* genes of *S. enterica* serovar Typhimurium and *P. aeruginosa* have been identified (39, 42). Since the original demonstration that IVET effectively identified *ivi* genes of *S. enterica* serovar Typhimurium, several other molecular techniques have been developed that could be used to identify *ivi* genes. These techniques include signature-tagged mutagenesis, subtractive hybridization, resolvase-dependent detection, and the use of patients serum antibodies that are absorbed with the cells of an in vitro-grown pathogen followed by screening of an expression library. By using these techniques, *ivi* genes of *Streptococcus pneumoniae*, *Streptococcus gordonii*, *A. pleuropneumoniae*, *S. aureus*, and *Mycobacterium avium* (10, 21, 24, 31, 32) have been isolated.

In this report we have described a variation of the IVET procedure to isolate *E. coli* genes that were induced in an infected host during bacterial septicemia. This method utilizes a promoter trap vector, pKK232-8, which carries a promoterless antibiotic resistance gene (for chloramphenicol acetyltransferase). In this *ivi* gene selection scheme, transcriptionally active cloned DNA fragments were selected in an antibiotic-treated mouse infection model. The fact that the modified IVET procedure identified several previously known virulence-associated genes, such as *aroA*, *clpA*, *fepE*, and *iucA* (Table 3), helped to validate the approach. A similar method has been successfully used to identify *ivi* genes in *S. enterica* serovar Typhi and *Y. enterocolitica* (38, 46). Another difference between the original IVET procedure and the one described here is that it was not necessary to integrate the hybrid plasmid into the chromosome. This simplifies the DNA sequencing of the cloned fragment.

The screening method used in this study selected for genes that were expressed during in vivo growth. To identify those expressed exclusively in vivo, a combination of in vivo and in vitro selection using chloramphenicol was used. By selecting in vivo chloramphenicol-resistant but in vitro chloramphenicol-sensitive clones, genes that were constitutively expressed regardless of growth conditions were eliminated. Initially, after in vivo selection, the pool of recovered bacterial clones was screened for in vitro resistance or sensitivity to chloramphenicol. Isolated bacterial cells were replica plated on LB agar either with chloramphenicol or with ampicillin. All of the clones expressing the reporter gene in vitro were excluded, while the remaining clones were subjected to additional in vivo selection. After three successive rounds of in vivo and in vitro selection, only genes that were believed to be exclusively expressed in vivo were identified. This conclusion was confirmed by demonstrating that the reporter gene transcript (*cat*) was found only in mice infected with one of the clones and not when cells were grown in LB medium.

Although the *ivi* genes identified here were expressed in infected mouse liver, it is possible that specific sets of *ivi* genes may be induced in other host tissues. The method we described could readily be adopted to identify genes induced in other tissues. Since chromosomal integration was not required in this procedure, analysis of the cloned sequence is simple, because no subcloning steps are required. In addition, the induction of the reporter gene can easily be detected in infected tissues by RT-PCR with this modified IVET procedure.

With this modified IVET procedure using the mouse model of septicemic infection, 750 *ivi*-specific colonies were isolated and 168 have been sequenced. The *E. coli ivi* genes identified can be grouped as follows: (i) genes that encode enzymes required for biosynthetic pathways, (ii) genes induced in response to DNA damage as part of the SOS response, (iii) unclassified ORFs with possible functions assigned based on some degree of homology to known genes, (iv) hypothetical ORFs of unknown function, and (v) ORFs bearing no known homology to any sequences in the public databases. Among the biosynthetic genes, *aroA*, which is required for aromatic amino acid biosynthesis, is well characterized. Mutations in *aroA* have been shown to result in attenuation in *S. enterica* serovar Typhi, *S. enterica* serovar Typhimurium, and *Neisseria gonorrhoeae* (5, 8, 19). In addition, an aromatic biosynthetic mutant of the fish pathogen *Aeromonas salmonicida* has been reported to be attenuated (41). The fact that *aroA* was detected provides supportive evidence that the procedure indeed could be used to identify essential genes expressed in vivo. In this study, the *aroA* mutation created in *E. coli* i484 was not significantly attenuated. The low levels of attenuation of *E. coli* strain i484 Δ*aroA* must have been due to the limitations of the competitive assay, in which a mixture of biosynthetic mutant and wild-type strains was used. Since chorismate is the precursor for enterobactin biosynthesis (14), the *aroA* mutant, which is unable to synthesize chorismate, could utilize enterobactin synthesized by the wild-type strain used for coinfection.

Another *ivi* biosynthetic gene, *metK*, encodes *S*-adenosylmethionine synthetase. MetK plays an important role in cellular metabolism. In *E. coli* and *S. enterica* serovar Typhimurium it acts as a corepressor of genes encoding methionine biosynthetic enzymes (33). *E. coli* mutants impaired in *S*-adenosylmethionine synthetase have been shown to cause constitutive expression of the heat shock protein LysU (28). In addition to *metK*, *E. coli* also has a second gene for *S*-adenosylmethionine synthetase, *metX* (34). Both *metK* and *metX* are differentially regulated. *metX* is expressed in rich medium while *metK* is specifically induced in minimal medium (34). This observation further suggests the *ivi* nature of *metK*, which may be induced in the nutritionally depleted environment of mouse liver during septicemic infection, but when an *ivi metK* clone was grown on rich LB agar it could not induce the reporter *cat* gene.

Two of the identified *E. coli ivi* ORFs, *o758* and *f443*, share 99 and 100% similarity with ATP-dependent proteases ClpB and HslV, respectively. Clp belongs to a highly conserved fam-

ily of ATP-dependent proteases. This protease degrades unstable proteins and consequently modulates gene expression (44). For *Bacillus subtilis* it has been shown that the Clp protease is important in modulating stress responses and in modulating stationary-phase responses (23). No significant loss of virulence by the *clpB* mutant of *E. coli* strain i484 was observed in the in vivo mouse challenge-competition assay. The other protease, Hsl, is a heat shock protein that has been reported to play an important role in reversing SOS-mediated inhibition of cell division. Hsl degrades SulA, an SOS response cell division inhibitor (20). The Hsl-mediated degradation of SulA has been shown in vitro as well as in *E. coli* cells grown in LB medium (35, 36). It could be that the selective expression of ATP-dependent proteases in vivo is important for the survival of pathogenic *E. coli* in mouse liver.

Although previously known genes were identified as *ivi* genes, they were unable to express a reporter (*cat*) gene in vitro on rich medium that contained 25 μg of chloramphenicol per ml but could express the reporter gene in the livers of chloramphenicol-treated mice. The presence of *cat* gene transcripts in the livers of mice challenged with clones carrying *bgl*, *aroA*, or *clp* genes suggested that growth conditions found in mouse liver selectively induce these genes whereas in vitro growth on rich media simply inhibits the induction of these genes. It could be that specific growth conditions may be required to express these *ivi* genes in vitro, such as growth on medium with limiting nutrients. In fact, replicating certain in vivo growth conditions, such as iron and nutrition starvation, oxygen tension, and pH (29) has resulted in the identification of virulence-associated genes of bacterial pathogens. However, due to insufficient knowledge of the growth milieu that a pathogen encounters in the infected host, it is presently not possible to express all of the *ivi* genes in vitro. Regardless of the in vitro expression of the *ivi* genes, functional characterization of *ivi* genes will help elucidate their importance in pathogenesis and will provide better understanding of the highly complex nature of host-pathogen interaction. Since in vivo bacterial growth in mouse liver presents bacteria with unique sets of challenges, the expression of known *ivi* genes during septicemia might play important roles in enhancing in vivo bacterial survival and pathogenicity.

While identifying known genes involved in pathogenesis helps us to understand the process, the most interesting results are from the identification of sequences of unknown function. Thirty-two *E. coli* ORFs of unknown function were identified, although 18 of these ORFs have been assigned putative functions based on DNA or amino acid sequence similarity. Fourteen other ORFs, while being known based on previous sequencing projects, have no known functions. Ten *ivi* clones had no homology to sequences in the public databases, including *E. coli* K-12 and *E. coli* O157:H7. These clones could encode functions that are required to cause septicemia. One of these clones, ivi817, was mutagenized, and the virulence of the mutant was tested in the mouse competitive model. The mutant was highly attenuated (~28-fold), demonstrating the importance of the gene during septicemia. Similar results were obtained using a mutant of strain ivi815, carrying a hypothetical ORF of *E. coli* with unknown function. This mutant was attenuated ~81-fold. Mutants with mutations in ORF *o761* and ORF V also were unable to survive effectively against the wild-type strain in an infection model. These results indicate that the uncharacterized *ivi* clones are important for bacterial virulence or in vivo survival. Therefore, this method has facilitated the identification of novel virulence-associated factors that may play crucial roles in *E. coli* pathogenesis. Future experiments to characterize these genes will truly reflect the importance of these gene products in causing disease and may provide novel targets to treat or control *E. coli* infection.

In summary, the isolated known *ivi* genes reported here encode various functions, from transport of macromolecules to metabolism, from DNA repair and replication to cell division, and from amino acid synthesis to polypeptide biosynthesis and degradation. Therefore, we believe that the *ivi* DNA sequences of clones that are collectively termed ORFs may encode many physiological functions to enable the pathogen to compete effectively in the tissues of infected hosts. Furthermore, DNA sequences of *ivi* clones that do not have homologues in the DNA databases are of utmost interest, as they may be required to cause septicemia. The IVET method we described here identified potentially new virulence factors that may be required for survival in liver tissues of the infected animal. The method can be used to identify tissue-specific *ivi* genes of pathogenic bacteria. The recovery of pathogens from the challenged animal at different time points and from various infected organs may also enhance our understanding of the infection process. The technique used allowed us to identify known sequences of unknown functions as well as totally novel sequences. The identification of these ORFs should lead to studies that will further enhance our understanding of disease and the growth and survival of pathogens in various habitats. Finally, among the *ivi* clones selected for further studies, when mutations were created in the cloned sequences, they served to attenuate mutants except for the *clpB* and *aroA* mutants. Coupled with the demonstration that the IVET technique does select for genes expressed exclusively in vivo, based on the results of RT-PCR, we believe that this tool will be important in furthering our understanding of disease and disease control.

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