

# Second-Generation Recombination-Based In Vivo Expression Technology for Large-Scale Screening for *Vibrio cholerae* Genes Induced during Infection of the Mouse Small Intestine

C. G. Osorio,<sup>1</sup> J. A. Crawford,<sup>2</sup> J. Michalski,<sup>2</sup> H. Martinez-Wilson,<sup>3</sup>  
J. B. Kaper,<sup>2</sup> and A. Camilli<sup>3\*</sup>

Programa de Microbiología y Micología, Facultad de Medicina, Universidad de Chile, Santiago, Chile<sup>1</sup>;  
Center for Vaccine Development and Department of Microbiology and Immunology,  
University of Maryland School of Medicine, Baltimore, Maryland<sup>2</sup>; and  
Department of Molecular Biology and Microbiology, Tufts University  
Medical School, Tufts University, Boston, Massachusetts<sup>3</sup>

Received 21 June 2004/Returned for modification 7 September 2004/Accepted 12 October 2004

**We have constructed an improved recombination-based in vivo expression technology (RIVET) and used it as a screening method to identify *Vibrio cholerae* genes that are transcriptionally induced during infection of infant mice. The improvements include the introduction of modified substrate cassettes for resolvase that can be positively and negatively selected for, allowing selection of resolved strains from intestinal homogenates, and three different *tnpR* alleles that cover a range of translation initiation efficiencies, allowing identification of infection-induced genes that have low-to-moderate basal levels of transcription during growth in vitro. A transcriptional fusion library of 8,734 isolates of a *V. cholerae* El Tor strain that remain unresolved when the vibrios are grown in vitro was passed through infant mice, and 40 infection-induced genes were identified. Nine of these genes were inactivated by in-frame deletions, and their roles in growth in vitro and fitness during infection were measured by competition assays. Four mutant strains were attenuated >10-fold in vivo compared with the parental strain, demonstrating that infection-induced genes are enriched in genes essential for virulence.**

Much remains to be learned about genes that the facultative pathogen *Vibrio cholerae* induces during infection and how their protein products function during the complex and dynamic process in which this pathogen adapts to the human small intestine. Several new methods have been developed that are helping us to explore this process, including in vivo expression technology (IVET), signature-tagged mutagenesis, microarray technology, differential fluorescence induction, in vivo-induced antigen technology, and real-time reverse transcription-PCR, among others. A specific IVET method, recombination-based IVET (RIVET), has been used previously to identify *V. cholerae* genes that are induced during infection of infant mice (1, 4). RIVET is very sensitive to low or transient expression of in vivo-induced (*ivi*) genes during infection and is therefore capable of identifying members of this potentially interesting class of genes. However, this sensitivity is also a double-edged sword, as some *ivi* genes have low-to-moderate levels of expression in vitro and will therefore be lost during library construction, i.e., there will be premature excision (resolution) of the selectable cassette in such strains. In the present study, we have developed a modified RIVET that can overcome this main disadvantage and used it as a large-scale screening method to identify *V. cholerae* genes that are tran-

scriptionally induced during infant mouse infection. Briefly, the new system incorporates two different resolvable cassettes that differ in the efficiency of excision, as well as three different alleles of the resolvase-encoding gene *tnpR* that have different efficiencies of translation initiation. These modifications extend the number of *V. cholerae* strains that are unresolved in vitro that can be generated in the final library. Additional modifications include the incorporation of positive selection for resolved strains, as well as a preselection step for eliminating strains partially resolved in vitro. These modifications increase the efficiency of the screening method and reduce the frequency of false positives, respectively.

## MATERIALS AND METHODS

**Plasmid constructions and antibiotics used.** All of the PCR primers used to construct plasmids are listed in Table 1. All of the plasmids and strains used are listed in Table 2. Unless stated otherwise, the antibiotics used in plates were ampicillin (AP) at 100  $\mu\text{g ml}^{-1}$ , rifampin (RIF) at 10  $\mu\text{g ml}^{-1}$ , and kanamycin (KM) at 50  $\mu\text{g ml}^{-1}$ . 5-Bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside (X-Gal) was used at 40  $\mu\text{g ml}^{-1}$ . New RIVET plasmids lacking extraneous sequences found in the original RIVET plasmid pIVET5 were constructed with genes and other functional sequences present in pIVET5 (2) or pGP704 (16). Briefly, as shown in Fig. 1, each contains an *oriR6K* origin that needs the host-encoded protein  $\pi$  (*pir* gene product) for replication, the broad-host-range mobilizable region *mobRP4*, *bla* for ampicillin resistance, a promoterless allele of *tnpR*, and a promoterless allele of the *Escherichia coli lacZ* gene. A transcriptional terminator and a multiple cloning site (MCS) were placed immediately 3' of *bla*. Just downstream of the MCS are the *tnpR* and *lacZ* genes, which are transcriptionally coupled. All of these sequences were amplified by PCR from pIVET5 and ligated to make pIVET5n. The PCR primer pairs used to amplify *bla*, *oriR6K*, *mobRP4*,

\* Corresponding author. Mailing address: Tufts University Medical School, 150 Harrison Ave., Boston, MA 02111. Phone: (617) 636-2144. Fax: (617) 636-0337. E-mail: Andrew.Camilli@TUFTS.EDU.

TABLE 1. Oligonucleotide PCR and DNA sequencing primers used in this study

Primer <sup>a</sup>	5'-3' sequence	Use
VC1500-F0	AGGGAAATCGACGCTGATTC	SOE PCR
VC1500-F1	CCTATACTGTTTAATGACAC	SOE PCR
VC1500-F2	GCTCCTATTGTAGGTTGTTTGAATGAGTCA	SOE PCR
VC1500-R1	AAACAACCTACAATAGGAGCCGCCATTAAC	SOE PCR
VC1500-R2	CTTGAGAGAAAATGGTCAAG	SOE PCR
VC2487-F0	AACATAGAGTGCATACCTAG	SOE PCR
VC2487-F1	GGAAGGATGTGTGATGAGAG	SOE PCR
VC2487-F2	AAAGCATTGTGACGTGAATATTAGTGTTTC	SOE PCR
VC2487-R1	ATTCACGTCACAAAATGCTTTTCTCCATTAC	SOE PCR
VC2487-R2	GCATAAACAACCTCATATTG	SOE PCR
VC0488-F0	AGTATCAGCAACGATTACAG	SOE PCR
VC0488-F1	CCTGTCACGAGTGATTGCTC	SOE PCR
VC0488-F2	GGAAGTTATGTAATGGCAATGCGCTGGATA	SOE PCR
VC0488-R1	ATTGCCATTACATAAACCCTCATTGTTAT	SOE PCR
VC0488-R2	TAGAAGCGTCTCTTAAACTG	SOE PCR
VC0622-F0	ATCTTACGCGGTAGACTATC	SOE PCR
VC0622-F1	TGGGAAGTCGCTTACTGCTC	SOE PCR
VC0622-F2	ACGTTTTTTGTAAAGCAATCCGCAAGCGAG	SOE PCR
VC0622-R1	GATTGCTTTACAAAAACGTGAGGAGAATG	SOE PCR
VC0622-R2	ATAATGGCTCTTTGCCTGAG	SOE PCR
VC0874-F0	GTCGTGATGTTGAACTGG	SOE PCR
VC0874-F1	CCATCGAACAAAACCATGACG	SOE PCR
VC0874-F2	TGGTGAATTACATGGGCTATCTCCTCTGG	SOE PCR
VC0874-R1	ATAGCCCATGTAATTCCACACTCATGAGG	SOE PCR
VC0874-R2	CTTTGCGATAACCGTTGATT	SOE PCR
VC0130-F0	GGTGTATGACCAAGCGCATC	SOE PCR
VC0130-F1	GAGCGAGACGCTTGAGCAAG	SOE PCR
VC0130-F2	CTTGCGTTACATAAAAGAGCTGATAAGCCAG	SOE PCR
VC0130-R1	TCAGCTCTTTATGTAACGCAAGACGC	SOE PCR
VC0130-R2	CGTTTCAACTCTCGCTGCTC	SOE PCR
VC2705-F0	CTGCGATTAAACGACTTGATG	SOE PCR
VC2705-F1	GCTATTAGGCTGCTAACCTAAG	SOE PCR
VC2705-F2	GTATTAACCTACATCGAGATGAAGGAAG	SOE PCR
VC2705-R1	CATCTCGATGTAAGTTAATACTGACTACTCAA	SOE PCR
VC2705-R2	GTGATGCTCCCTGCATTG	SOE PCR
VC2646-F0	GAGATCATGCACGAAGTGCTG	SOE PCR
VC2646-F1	AATTTGTGGTTGCTGCGAAG	SOE PCR
VC2646-F2	ACTCACATTGTAACAAAATCGCTAAAATGTCA	SOE PCR
VC2646-R1	CGATTTGTTACAATGTGAGTTTCAGTTAATTACAC	SOE PCR
VC2646-R2	TTGAGTTCACGATATACTGT	SOE PCR
BLA-F	CGCGGAACCCCTATTTGT	pIVETn construction
BLA-R	GTCTGACAGTTACCAATG	pIVETn construction
ORI-F	CGCTAGTTTGTTTTGACT	pIVETn construction
ORI-R	CAGCAGTTCAACCTGTTG	pIVETn construction
MOB-F	CAGCCGACCAGGCTTTCC	pIVETn construction
MOB-R	CTTTTTGTCCGGTGTGG	pIVETn construction
TNPR-F	CGACCCGGGAGATCTCAATTGTTTCGAATTTAGGATACATTTTTAT	pIVETn construction
TNPR-R	GCGGTACCTTATGTTAGTTGCTTTCA	pIVETn construction
LACZ-F	GCGGTACCACAGGAAACAGCTATGACC	pIVETn construction
LACZ-R	TTATTATTTTTGACACCA	pIVETn construction
TNPRWTF4	GAGAATTTAGGATAAAATTTTTATGCGACTTTTTTGTTACG	pIVETn construction
TNPR135F5	GAGAATTTGAGATAAAATTTTTATGCGACTTTTTGGTTAC	pIVETn construction
TNPR168F6	GAGAATTTAAGATAAAATTTTTATGCGACTTTTTTGTTACG	pIVETn construction
TNPR-R4	ACAATTGAGATCTCGAGAAAAAGC	pIVETn construction
NEO-F	CGCTCTAGAGGCCTGTGTG	Cassette construction
NEO-R	CTGAGATCTAATGCTCTGCCAGTGTTAC	Cassette construction
IRGA-F	ATCTTGCATAGGTATTTGACC	P <sub>irgA</sub> cloning
IRGA-R	GCTGGCGCATTTTGAATCA	P <sub>irgA</sub> cloning
SACB1	GGAAGATCTGATCCTTTTTTAACCCATCATATATA	Cassette construction
SACB-R1	CTTCTAGAAAAGGTTAGGAATACGGTTAG	Cassette construction
1RES-F	TCTATTGAATFCCGTCCGAAATATTATAAATTATCGCAC	Cassette construction
1RES-R	TCTAATCTCGAGTGTATCCTAAATCAAATATCGGACAAG	Cassette construction
1RES1-F	TCTAATGAAATFCCGTCCGAAATATTACAAATTATCGCAC	Cassette construction
2RES-F	TCTAATCTCGAGTCTAGACGTCCGAAATATTATAAATTATCGCAC	Cassette construction
2RES1-F	TCTAATCTCGAGTCTAGACGTCCGAAATATTACAAATTATCGCAC	Cassette construction
2RES-R	TCTAATGGTACCTGTATCCTAAATCAAATATCGGACAAG	Cassette construction
VIVI-F	GACAGCGTGGCACAAAAAAGAGAG	Cassette confirmation
VIVI-R	ACCAAACTCACCCATCCGAGAG	Cassette confirmation
T3	AATTAACCCTCACTAAAGGG	Cassette integration
T7	AATACGACTCACTATAGGGC	Cassette integration

<sup>a</sup> Primers for VCA1008 SOE PCR are described in reference 18.

TABLE 2. Strains and plasmids used in this study

Strains or plasmid	Relevant genotype or phenotype	Source or reference
<i>V. cholerae</i>		
CVD110	E7946 El Tor Ogawa $\Delta(cep-ctxB)$ <i>hlyA::mer::ctxB</i> Hg <sup>r</sup>	15
GOA1264	Spontaneous Rif <sup>r</sup> form of CVD110 Rif <sup>r</sup>	This work
GOA1245	GOA1264 <i>res</i> cassette Rif <sup>r</sup> Km <sup>r</sup> Suc <sup>s</sup>	This work
GOA1246	GOA1264 <i>resI</i> cassette Rif <sup>r</sup> Km <sup>r</sup> Suc <sup>s</sup>	This work
GOA6W	GOA1264 <i>lacZ::pGP704</i> Ap <sup>r</sup> LacZ <sup>-</sup>	Laboratory strain
GOA1745	GOA1245 <i>irgA::pGOA1196</i> Rif <sup>r</sup> Km <sup>r</sup> Suc <sup>s</sup>	This work
GOA1746	GOA1245 <i>irgA::pGOA1197</i> Rif <sup>r</sup> Km <sup>r</sup> Suc <sup>s</sup>	This work
GOA1747	GOA1246 <i>irgA::pGOA1196</i> Rif <sup>r</sup> Km <sup>r</sup> Suc <sup>s</sup>	This work
GOA1748	GOA1246 <i>irgA::pGOA1197</i> Rif <sup>r</sup> Km <sup>r</sup> Suc <sup>s</sup>	This work
GOA1737	GOA1264 $\Delta$ VVC1500 Rif <sup>r</sup>	This work
GOA1738	GOA1264 $\Delta$ VVC2487 Rif <sup>r</sup>	This work
GOA1739	GOA1264 $\Delta$ VVC0488 Rif <sup>r</sup>	This work
GOA1740	GOA1264 $\Delta$ VVC0622 Rif <sup>r</sup>	This work
GOA1741	GOA1264 $\Delta$ VVC0874 Rif <sup>r</sup>	This work
GOA1742	GOA1264 $\Delta$ VVC0130 Rif <sup>r</sup>	This work
GOA1743	GOA1264 $\Delta$ VVC2705 Rif <sup>r</sup>	This work
GOA1744	GOA1264 $\Delta$ VVC2646 Rif <sup>r</sup>	This work
<i>E. coli</i>		
SM10 $\lambda$ <i>pir</i>	<i>thi recA thr leu tonA lacY supE</i> RP4-2-Tc::Mu $\lambda::pir$ Km <sup>r</sup>	Laboratory strain
DH5 $\alpha$ <i>pir</i>	F <sup>-</sup> $\phi$ 80 $\Delta$ lacZ $\Delta$ M15 $\Delta$ (lacZYA-argF)U169 <i>recA1 endA1 hsdR1 supE44 thi-1 gyrA96 relA1::pir</i>	10
Plasmids		
pRR51	pBR322::( <i>res-tet-res</i> )	20a
pGP704	<i>oriR6K mobRP4</i> Ap <sup>r</sup>	16
pAC212	pGP704::neo	Laboratory plasmid
pCVD442	<i>oriR6K mobRP4 sacB</i> Ap <sup>r</sup> Suc <sup>s</sup>	5
pSL134	pPCR-Script::( <i>resI-resI</i> )	Laboratory plasmid
pGOA1	pSL134 <i>lacZ::(resI-neo-sacB-resI)</i>	This work
pGOA2	pPCR-Script::( <i>res</i> )	This work
pGOA3	pPCR-Script::( <i>resI</i> )	This work
pGOA4	pPCR-Script::( <i>res-res</i> )	This work
pGOA5	pPCR-Script::( <i>resI-resI</i> )	This work
pGOA6	pGOA4::neo-sacB	This work
pGOA7	pGOA5::neo-sacB	This work
pSL111	pGP704::lacZ	Laboratory plasmid
pIVET5	<i>oriR6K mobRP4 lacZ tnpR</i> Ap <sup>r</sup>	2
pIVET5n	pIVET5 derivative	This work
pRES	pSL111::( <i>res-neo-sacB-res</i> )	This work
pRES1	pSL111::( <i>resI-neo-sacB-resI</i> )	This work
pGOA1193	pIVET5n <i>tnpR</i>	This work
pGOA1194	pIVET5n <i>tnpR</i> <sup>mut168</sup>	This work
pGOA1195	pIVET5n <i>tnpR</i> <sup>mut135</sup>	This work
pGOA1196	pGOA1193::P <sub><i>irgA</i></sub>	This work
pGOA1197	pGOA1195::P <sub><i>irgA</i></sub>	This work

*tnpR*, and *lacZ* were BLA-F and BLA-R, ORI-F and ORI-R, MOB-F and MOB-R, TNPR-F and TNPR-R, and LACZ-F and LACZ-R, respectively. All PCRs used *Pfu* polymerase (Stratagene). pIVET5n was used as the template to construct three derivatives altered in the *tnpR* ribosome-binding sequence (RBS). Plasmid derivatives pGOA1193, pGOA1194, and pGOA1195 were thus made by PCR with primers TNPR-F4 and TNPR-R4, TNPR-F6 and TNPR-R4, and TNPR-F5 and TNPR-R4, respectively. After amplification, the PCR products were circularized by intramolecular ligation. Each of these primer pairs incorporated stop codons in all three frames between the MCS and *tnpR* plus one of three different RBSs for *tnpR*. The RBSs for *tnpR*(pGOA1193), *tnpR*<sup>mut168</sup>(pGOA1194), and *tnpR*<sup>mut135</sup>(pGOA1195) are TTTAGGA, TTAAAGA, and TTTGAGA, respectively. The entire DNA sequence of pGOA1193 and the *tnpR* RBSs of pGOA1194 and pGOA1195 were determined to confirm their correct structure (data not shown).

**Construction of *V. cholerae* transcriptional fusion libraries.** The strain used to construct the library, designated GOA1264, was a spontaneously rifampin-resistant (Rif<sup>r</sup>) strain derived from E7946 El Tor Ogawa derivative CVD110. A resolvable cassette, illustrated in Fig. 1, incorporating the *neo* and *sacB* genes

flanked by *res* or *resI* sequences was inserted into the *lacZ* locus of GOA1264 by allelic exchange as described previously (2). To construct the cassette, the *neo* gene was PCR amplified from plasmid pAC212 with primers NEO-F and NEO-R, and *sacB* was PCR amplified from pCVD442 with primers SACB1 and SACB1R1. The products were ligated into pSL134 to generate pGOA1. The *res* and *resI* sequences were PCR amplified from pRR51 and pSL134 with primers 1RES-F and 1RES-R and primers 1RES1-F and 1RES1-R, respectively. The *res* and *resI* products were cloned into pPCR-Script Amp SK(+) (Stratagene) to generate plasmids pGOA2 and pGOA3, respectively. Next, a second set of primers, 2RESF and 2RESR or 2RES1F and 2RES1R, was used to PCR amplify a second copy of *res* or *resI*. These were inserted immediately next to their counterparts in pGOA2 and pGOA3 to generate plasmids pGOA4 and pGOA5, in which the *res* and *resI* sequences are directly repeated. The *sacB-neo* genes were subcloned from pGOA1 into XbaI-digested pGOA4 and pGOA5 to generate pGOA6 and pGOA7, respectively. The *res* and *resI* cassettes were PCR amplified from pGOA6 and pGOA7 with primers T3 and T7 and ligated into KpnI-digested pSL111 treated with T4 DNA polymerase. The final plasmids were designated pRES and pRES1, and their structures were confirmed by DNA

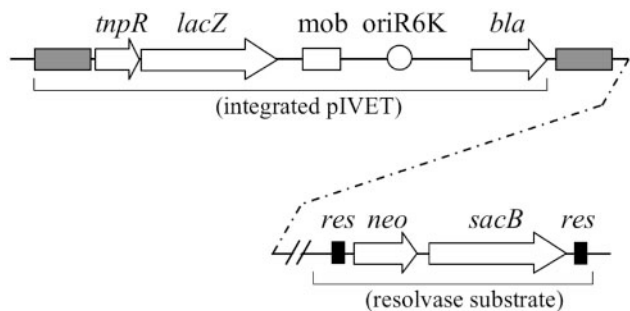


FIG. 1. Schematic diagram of the second-generation RIVET method. A hypothetical integration of pGOA1193 into the genome to generate a merodiploid is shown along with one of the two resolvase substrate cassettes used in this study (the *res* version is shown). In the example shown, the two regions of the genome are unlinked, which is indicated by the dashed line. Genes are shown by open arrows and are labeled (*tnpR*, resolvase; *lacZ*,  $\beta$ -galactosidase; *bla*, ampicillin resistance; *neo*, kanamycin resistance; *sacB*, sucrose sensitivity). The two other plasmids used in this study, pGOA1194 and pGOA1195, are identical to pGOA1193, except for alterations in the *tnpR* ribosome-binding sequence. The *res* sequences, which are recognized by resolvase, are shown as filled rectangles. The mobilization (*mob*) and origin of replication (*oriR6K*) are shown by the open rectangle and circle, respectively. The genomic fragment that was cloned into the pIVET derivative and allowed homologous recombination into the genome is shown by the gray filled rectangle. Note that in the merodiploid product of this recombination, this fragment is duplicated downstream of the integrated plasmid as shown.

sequencing (data not shown). pRES and pRES1 were mobilized into strain GOA1264 by conjugation, and allelic exchange was used to place each cassette into the *lacZ* locus as previously described (2). The *res* and *res1* cassette-containing strains were designated GOA1245 and GOA1246, respectively. The correct placement of the resolvase cassettes was confirmed by PCR with primers VIVIF and VIVIR, which flank the *lacZ* gene.

Libraries of *V. cholerae* genomic fragment fusions to *tnpR* were made in *E. coli* SM10 $\lambda$ pir. The genome of GOA1264 was partially digested with either restriction enzyme Sau3AI or Tsp509I, and then the fragments between 1.5 and 3 kb were purified from agarose gels. pGOA1193, pGOA1194, and pGOA1195 were digested with BglII or MfeI, which yield compatible cohesive ends with Sau3AI and Tsp509I, respectively. The digested plasmids were dephosphorylated with calf intestinal phosphatase (New England Biolabs) and purified from agarose gels. The genomic fragments were ligated into the BglII and MfeI sites upstream of the promoterless resolvase allele in each of the digested plasmids. Thus, a total of six separate ligations were done. Each ligated DNA was electroporated into *E. coli* DH5 $\alpha$ pir, the transformed cells were incubated for 1 h at 37°C in Luria-Bertani (LB) broth, and then each was plated for selection of transformants on LB agar supplemented with AP at 50  $\mu$ g ml<sup>-1</sup>. Approximately 15,000 transformants were pooled from each of the six ligation combinations. Plasmid DNA was purified en masse from each of the six pools and electrophoresed on agarose gels. Recombinant plasmids 8.5 to 10 kb in size were purified from the gels, electroporated into *E. coli* SM10 $\lambda$ pir, and after 1 h of growth at 37°C in LB broth, cells were plated for selection of Ap<sup>r</sup> transformants on LB agar supplemented with AP at 50  $\mu$ g ml<sup>-1</sup>. Approximately 20,000 transformants were pooled from each of the six electroporations and stored at -70°C in 10% glycerol.

**Construction of *irgA-tnpR* fusion strains.** To generate *V. cholerae* strains with transcriptional fusions of *irgA* to two *tnpR* alleles, a genomic fragment containing the *irgA* promoter was PCR amplified with primers IRGA-F and IRGA-R, which contain BglII restriction sites in their 5' ends. The amplification products were digested with BglII, and then each was ligated separately into the BglII sites in pGOA1193 and pGOA1195 as previously described (12). The resulting plasmids were mobilized into GOA1245 and GOA1246 by conjugation. Insertion-duplication into the *irgA* locus was confirmed by PCR (data not shown). Resolution of the resulting reporter strains was measured after growth in LB broth containing a range of ferrous iron concentrations as previously described (12).

**Preselection of fusion strains that are inactive in vitro.** Each of the six *E. coli* SM10 $\lambda$ pir plasmid libraries was mobilized into GOA1245 and GOA1246 by conjugation at 37°C for 1 h on AAWP02500 filters (Millipore) on the surface of

LB agar plates. Thus, a total of 12 matings were done. Unresolved transconjugants were selected on LB agar supplemented with RIF, AP, KM, and X-Gal. Eleven thousand six hundred sixteen colonies were isolated by picking individual light blue ones with sterile toothpicks. The light blue colonies harbor *tnpR-lacZ* fusions that are inactive or expressed at a low-level (data not shown). The 11,616 colonies were composed roughly equally of strains from each of the 12 matings. Each colony was grown individually in microtiter plate wells in 100  $\mu$ l of LB broth plus RIF at 10  $\mu$ g ml<sup>-1</sup>, AP at 50  $\mu$ g ml<sup>-1</sup>, and KM at 25  $\mu$ g ml<sup>-1</sup> at 37°C for 4 to 5 h and then incubated overnight at room temperature.

A second preselection step to specifically eliminate partially resolved strains was done. For this, each of the 11,616 strains in microtiter plate wells was diluted 1:2,000 in 2 ml of LB broth in deep-well microtiter plates. Five microliters from each diluted culture was spotted onto rectangular LB agar plates plus RIF and also onto LB agar lacking NaCl plus RIF and 10% sucrose. The plates were incubated for 16 h at 30°C, and then the approximate percent resolution of each strain was determined by dividing the number of colonies on the RIF-sucrose plate by the number of colonies on the RIF plate. All strains determined to have resolved to approximately 1% or less were pooled from the microtiter plates, and aliquots were frozen at -70°C in 20% glycerol. The final transcriptional fusion library consisted of 8,734 strains of *V. cholerae*, each harboring a fusion that was inactive or active at a low level when cells were grown in vitro in LB broth.

**Screening for *V. cholerae* *ivi* genes.** An aliquot of the library was thawed and grown for approximately 10 generations at 37°C in LB broth plus RIF at 10  $\mu$ g ml<sup>-1</sup>, AP at 50  $\mu$ g ml<sup>-1</sup>, and KM at 25  $\mu$ g ml<sup>-1</sup> to late exponential phase. The cells were then diluted 1:10 in LB broth and used to intragastrically inoculate 5-day-old CD-1 mice with 50  $\mu$ l (approximately  $5 \times 10^6$  CFU) as previously described (2). After 24 h, the mice were euthanized, their small intestines were removed and homogenized in 2 ml of LB broth plus 20% glycerol, and serial dilutions were plated on LB agar minus NaCl plus 10% sucrose and RIF. The plates were incubated for 16 h at 30°C. Individual colonies were picked and grown in LB broth plus RIF at 10  $\mu$ g ml<sup>-1</sup> and AP at 50  $\mu$ g ml<sup>-1</sup>. The integrated plasmids were recovered from these strains as previously described (2). Approximately 200 bases of *V. cholerae* DNA flanking the 5' end of *tnpR* was sequenced from each recovered plasmid by arbitrary primed PCR and DNA sequencing as previously described (14). The flanking sequences were compared to the *V. cholerae* N16916 genome database with blastN (<http://tigrblast.tigr.org/cmrr-BLAST/index.cgi?database=GVC.seq>). The sequence information obtained also revealed which *tnpR* allele was present in the fusion.

**Fusion strain reconstructions and quantification of resolution.** The recovered plasmids of interest were concentrated by ethanol precipitation and resuspension in 10  $\mu$ l of deionized water. Each plasmid was electroporated into *E. coli* DH5 $\alpha$ pir and then selected on LB agar plus AP at 50  $\mu$ g ml<sup>-1</sup> and X-Gal. For each plasmid, one blue colony was selected and inoculated into LB broth plus AP at 50  $\mu$ g ml<sup>-1</sup>. Note that the basal level of plasmid *lacZ* expression is high in this *E. coli* host and the rarely observed white and light blue colonies were found to harbor spontaneous deletions of large portions of the plasmid including part or all of *lacZ* (data not shown). Plasmid DNA was prepared from each *E. coli* DH5 $\alpha$ pir transformant strain and electroporated into *E. coli* SM10 $\lambda$ pir with selection for Ap<sup>r</sup> transformants. Approximately 50 to 200 transformant colonies were pooled from each electroporation and mated with either GOA1245 or GOA1246, followed by selection for unresolved transconjugants as described above. The extent of resolution of each strain after growth in LB broth and after infection of infant mice was measured as previously described (2), except that the percent resolution for each strain was calculated by dividing the titer on LB agar minus NaCl plus RIF and 10% sucrose by the titer on LB agar plus RIF.

**Construction of deletion mutation strains.** In-frame deletions of the entire coding sequence of selected *V. cholerae* *ivi* genes were constructed in pCVD442 by splicing by overlap extension (SOE) PCR (21) with the oligonucleotide primer pairs in Table 1. Each recombinant pCVD442 plasmid was electroporated into *E. coli* SM10 $\lambda$ pir and transferred to *V. cholerae* strain GOA1264 by conjugation. Allelic exchange was done as previously described (5), and the chromosomal deletion mutations were confirmed by PCR with primers F0 and R2 (Table 1), followed by DNA sequencing (data not shown).

**Competition assays.** Each of the *ivi* gene deletion strains of *V. cholerae* (naturally LacZ<sup>+</sup>) was tested for virulence by competition assays against isogenic LacZ<sup>-</sup> wild-type strain GOA6W. The GOA6W strain contains a pGP704 insertion in the *lacZ* locus. Each deletion strain and GOA6W were grown to mid-exponential phase in LB broth plus RIF at 10  $\mu$ g ml<sup>-1</sup> and mixed 1:1. Approximately 10<sup>5</sup> CFU were inoculated intragastrically into 8 to 10 infant mice as previously described (2). In vitro competition assays were done in parallel by using each of the prepared inocula to inoculate 2 ml of LB broth with 10<sup>4</sup> CFU; after which the cultures were grown for 16 h at 37°C with aeration, representing approximately 18 generations. The ratio of the test strain to the wild-type strain



in each inoculum, as well as in the resulting bacterial populations recovered from the in vitro and in vivo competition assays after 16 and 24 h, respectively, was determined by plating serial dilutions of the outputs on LB agar plus RIF at  $10 \mu\text{g ml}^{-1}$  and X-Gal at  $40 \mu\text{g ml}^{-1}$ . The in vivo and in vitro competitive indices were calculated by dividing the in vivo or in vitro output ratios of the test strain to GOA6W by the respective input ratios.

## RESULTS

**Improvements of RIVET.** The original version of RIVET, which was applied to the discovery of *V. cholerae* *ivi* genes, used transcriptional fusions to a promoterless resolvase gene (*tnpR*) in order to couple transcriptional induction to excision of a tetracycline resistance cassette flanked by TnpR recognition sequences (*res*) (2). The excision of the cassette, being irreversible, led to a phenotypic change (tetracycline sensitivity) that could subsequently be screened for by replica plating.

The RIVET screening method used in the present study incorporates three modifications with respect to the original RIVET method. First, the gene cassette that is the target for TnpR has two reporter genes, *neo* and *sacB*, instead of a gene for tetracycline resistance (Fig. 1). The *neo* gene confers resistance to the antibiotic KM. The *sacB* gene encodes the levansucrase from *Bacillus subtilis*. This enzyme produces toxic products in the presence of sucrose and as a result prevents *V. cholerae* from growing on 10% sucrose plates. Thus, when the *sacB-neo* genes are excised from the genome by the action of TnpR, the resolved strain becomes sensitive to kanamycin and resistant to sucrose, allowing positive selection. Second, the screening method incorporates three different alleles of the *tnpR* gene in the pIVET plasmid (Fig. 1) that differ in translation efficiency. Because many *ivi* genes have low-to-moderate levels of expression in vitro, they require a less active *tnpR* allele to remain unresolved prior to infection. This modification allows an increase in the number and variety of fusion strains that make up the final library. The third modification was to incorporate a prescreening step to eliminate fusion strains with low levels of resolution in vitro but that were nevertheless still capable of growing on media containing KM. Briefly, the prescreening method involved growing each fusion strain overnight to stationary phase in LB broth in the absence of KM selection and then plating serial dilutions on both LB agar and LB agar supplemented with sucrose. After overnight incubation of the plates, the bacterial titer on sucrose plates was divided by the titer on LB agar in order to calculate the percent resolution that occurred during the growth of each strain in broth. We collected only those fusion strains that resolved to  $\leq 0.1\%$  for inclusion in our library. As will become evident below, these three modifications have greatly improved the efficiency and accuracy of the screening method.

The *V. cholerae* strain used in our screening method is a derivative of strain CVD110 (E7946 El Tor Ogawa). This toxin-deficient strain has been used before for human vaccine trials and was very efficient at colonizing the human intestine compared with other vaccine strains (15). After genetically modifying this strain (rifampin resistance and *res* cassette incorporation), we tested if its ability to colonize the infant mouse small intestine was maintained. In single-strain infections, its level of colonization after 24 h ( $2 \times 10^6$  CFU) was equivalent to that of parental strain CVD110 ( $8 \times 10^5$  CFU).

Another preliminary experiment done was to assay the in

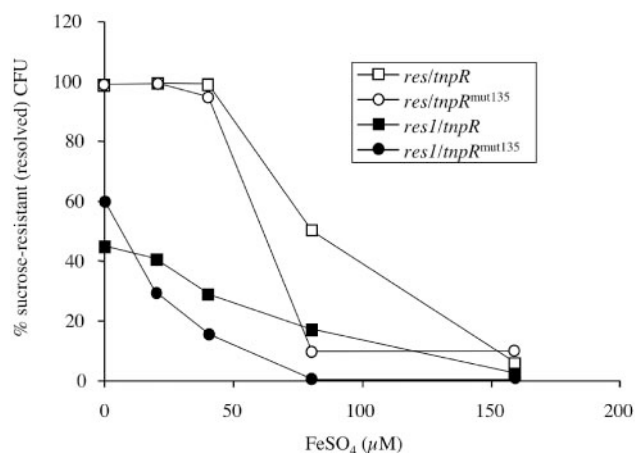


FIG. 2. Resolution levels versus iron concentration for four *V. cholerae* reporter strains harboring an iron-repressed fusion to *tnpR*. Symbols: open squares, GOA1745 (*lacZ::res-neo-sacB-res irgA::tnpR*); open circles, GOA1746 (*lacZ::res-neo-sacB-res irgA::tnpR<sup>mut135</sup>*); solid squares, GOA1747 (*lacZ::res1-neo-sacB-res1 irgA::tnpR*); solid circles, GOA1748 (*lacZ::res1-neo-sacB-res1 irgA::tnpR<sup>mut135</sup>*).

vitro resolution efficiencies of our final *V. cholerae* reporter strain constructs with the iron-repressible *irgA* promoter transcriptionally fused to *tnpR* in two versions of the RIVET plasmid, pGOA1193 and pGOA1195. The extent of resolution was measured after 8 h of growth in LB broth containing various concentrations of ferrous iron. As the concentration of ferrous iron decreased, level of resolution for each of the strains increased, although with a different dose response for each strain (Fig. 2). Strains GOA1745 and GOA1746, harboring the more sensitive *res* cassette, resolved more readily than the respective isogenic strains GOA1747 and GOA1748, which contained the *res1* mutant cassette. Likewise, strains GOA1745 and GOA1747, harboring the higher translational efficiency *tnpR* allele, resolved more readily than the respective isogenic strains GOA1746 and GOA1748, which contained the less efficient *tnpR<sup>mut135</sup>* allele. These dose responses are in accordance with the expected levels of resolution previously reported for similar strain constructs (12).

**Screening for *V. cholerae* *ivi* genes.** The final *V. cholerae* *tnpR* fusion strain library contained 8,734 strains each having no or only a very low level of resolution after growth in LB broth. To assess the randomness of the library, we recovered the integrated plasmids from 200 randomly selected strains and sequenced the fusion joints as described in Materials and Methods. Sequences flanking the 5' end of *tnpR* were compared to the *V. cholerae* genome (7) with the blastN algorithm. The results indicated that 95% of the *V. cholerae* fusion strains are unique.

We inoculated the library intragastrically into 120 infant mice and, after infection for 24 h, plated serial dilutions of small intestinal homogenates on sucrose plates. We isolated a total of 384 sucrose-resistant (resolved) strains on sucrose plates. We attempted to recover the integrated plasmid from each strain and sequence the fusion junctions as described in Materials and Methods. Briefly, the nonreplicating plasmid is able to excise from the chromosome at a low frequency. As a result, the plasmid can be captured by plasmid DNA purification and electroporation into an *E. coli* strain that supports

plasmid replication. Such captured plasmids retain the original *V. cholerae* genomic DNA insert fused to *tnpR*. We failed to recover the plasmid or obtain the DNA sequence of the fusion junction for 43 of these, whereas the remainder comprised 122 unique junctions. Ninety-four of the 122 unique gene fusions were plus strand fusions (the *tnpR* gene was in the same transcriptional orientation as the gene into which it was integrated). We reconstructed 47 of the plus strand fusions into the unresolved GOA1245 and GOA1246 strain backgrounds (*res* and *res1* cassettes, respectively). Each reconstructed strain was tested for resolution in vitro (growth in LB broth) and in vivo (infant mouse infection). Forty (85%) of the 47 reconstructed fusion strains resolved to a greater extent during infection than during growth in vitro and thus contained bona fide *ivi* genes (Table 3).

***V. cholerae* *ivi* gene functions.** Since we only reconstructed a subset of the original 122 unique gene fusions recovered from mice, the composition of the final confirmed set of *ivi* genes in terms of gene categories was altered by our subjective selection. For example, one-third of the unique plus strand fusions were in the conserved/hypothetical category but only 10% of our confirmed genes were in this category. In addition, since we decided to study only plus strand fusions, we have undermined the potential role of putative regulatory RNAs in virulence (see Discussion). This notwithstanding, many (15%) of the confirmed *ivi* genes were previously identified by screening for *ivi* genes (2; A. Camilli, D. Merrell, and M. Angelichio, unpublished data) or for essential virulence genes (3, 14).

Among the confirmed *ivi* genes identified are three genes related to chemotaxis: VCA0773 (methyl-accepting chemotaxis protein), VC1535 (methyl-accepting chemotaxis protein), and VC2130 (*ftii*; flagellum-specific ATP synthase). A large number of metabolic genes, 13, were identified: VC2641 (*argH*; argininosuccinate lyase), VC1111 (*bioA*; adenosylmethionine-8-amino-7-oxononanoate aminotransferase), VC1173 (*trpG*; anthranilate synthase component II), VC1338 (*acnA*; aconitase hydratase 1), VC1137 (*hisA*; phosphoribosylformimino-5-aminoimidazole carboxamide ribotide isomerase), VCA1057 (oxidoreductase short-chain dehydrogenase), VC2373 (*gluB1*; glutamate synthase, large subunit), VCA0016 (*glgB*; 1,4- $\alpha$ -glucan branching enzyme), VCA0014 (*malQ*; 4- $\alpha$ -glucanotransferase), VC1034 (*udp-I*; uridine phosphorylase), VCA0242 (*sgbH*; putative hexulose-6-phosphate synthase), VC2646 (*ppc*; phosphoenolpyruvate carboxylase), and VC0063 (*thiF*). Of these genes and pathways, *bioB*, *trpG*, and *malQ* were previously identified as essential virulence genes (3, 14) while *argA* and VCA0773 were identified as *ivi* genes (2; Camilli et al., unpublished).

One of the 41 GGDEF domain-containing proteins encoded in the *V. cholerae* genome, the product of gene VC0130, is induced in mice (Table 3). GGDEF domains are believed to catalyze synthesis of cyclic diguanylate (c-di-GMP). Recently, a role in virulence for the *V. cholerae* VieA protein, which can degrade c-di-GMP, was reported (23, 23a), suggesting that c-di-GMP signaling plays an important regulatory role in virulence.

The second largest class of genes identified as *ivi* genes are the 12 involved in transport across the envelope: VC1008 (putative outer membrane protein), VC0488 (putative extracellular solute-binding protein), VC1275 (conserved protein similar

to proteins from the TRAP transport systems), VC0207 (PTS system, sucrose-specific IIBC component), VC0201-0202-0203 [iron(III) ABC transporter components; ATP-binding protein, periplasmic iron compound-binding protein, and permease protein, respectively], VCA0687 [iron(III) ABC transporter, ATP-binding protein], VC1658 (*sdaC2*; serine transporter), VC2705 (putative sodium solute symporter), VCA0529 (*kup*; potassium uptake protein, Kup system), and VC0721 (phosphate ABC transporter, putative periplasmic phosphate-binding protein).

Three of the *ivi* genes may be involved in sensory systems: VC0622 (putative sensory box sensor histidine kinase/response regulator), VCA0752 (*trxC*; thioredoxin 2), and VC1500 (PqiA family protein).

One putative RTX toxin was identified, the product of the VC1619.1 gene. This protein could have important roles during infection, like many previously described RTX toxins (6, 11).

Four *ivi* genes are predicted to be involved in various aspects of nucleic acid metabolism, including VC2419 (*xerD*; integrase/recombinase protein), VCA0632 (MutT/nudix family protein), VC2621 (extracellular nuclease-related protein), and VC2742 (*rbn*; RNase BN). The extracellular nuclease gene VC2621 was previously identified as an *ivi* gene (Camilli et al., unpublished).

Finally, there were a number of hypothetical conserved *ivi* genes (3), including VC2487 (encoding a product similar to glycosyltransferases), VC0874 (hypothetical), and VC1687 (encoding a product similar to inorganic pyrophosphatase/exopolyphosphatase).

**Competition assays of *V. cholerae* mutant strains in vitro and in infant mice.** Some *ivi* genes are likely to play important roles during colonization but may be redundant, while others may play essential, nonredundant roles. To determine if some of the identified *ivi* genes fall into the latter class, we made in-frame deletions of the entire coding sequence of nine *ivi* genes and tested each strain by competition assay during in vitro growth and infant mouse infection. The VCA1008 deletion strain was tested as part of a separate study of *V. cholerae* outer membrane porins (18); however, it should be noted that its level of attenuation in vivo was found in that study to be 40-fold but with no detectable growth defect in vitro. In this study, three deletion strains showed attenuation levels of more than 10-fold (Table 4). These are strains with deletions in VC2487, VC0874, and VC2705, which were attenuated 20-, 22-, and 14-fold, respectively, in vivo. Our attempt to make an in-frame deletion of VC1619.1 (encoding a putative RTX toxin) was unsuccessful, and this was not rigorously pursued.

## DISCUSSION

The IVET methods are the only established ones for large-scale screening for *ivi* genes within infected animal tissues. Although transcriptional profiling by DNA microarrays has been used to measure gene expression averages within in vivo populations of *V. cholerae*, it has thus far required compromising the anatomy of the animal by closing off sections of small intestine with ligatures (ileal-loop model) (24). Of the IVET methods, the RIVET system has unique advantages, primarily its sensitivity to transient and/or low-level gene expression, as

TABLE 3. *V. cholerae* gene products identified by RIVET as being transcriptionally induced during infection of the infant mouse small intestine compared to during growth in LB broth

Gene product <sup>a</sup> :	Cassette <sup>b</sup>	Allele <sup>c</sup>	% of CFU resolved		Ratio <sup>f</sup>	No. of mice <sup>g</sup>	P value <sup>h</sup>
			In vitro <sup>d</sup>	In vivo <sup>e</sup>			
VCA1008	<i>res</i>	<i>tnpR</i> <sup>mut135</sup>	<0.1	77	770	4	<0.05
VC2487	<i>res1</i>	<i>tnpR</i> <sup>mut168</sup>	<0.1	72	720	4	<0.05
VC2641	<i>res1</i>	<i>tnpR</i> <sup>mut135</sup>	<0.1	70	700	4	<0.05
VC0488	<i>res</i>	<i>tnpR</i> <sup>mut168</sup>	<0.1	22	221	4	<0.05
VC0063	<i>res1</i>	<i>tnpR</i> <sup>mut135</sup>	0.1	22	220	4	<0.05
VCA1057	<i>res</i>	<i>tnpR</i> <sup>mut135</sup>	0.3	35	117	4	<0.05
VCA0773	<i>res1</i>	<i>tnpR</i> <sup>mut168</sup>	1	100	100	7	<0.05
VC0622	<i>res1</i>	<i>tnpR</i> <sup>mut168</sup>	0.1	10	100	4	<0.05
VC1111	<i>res</i>	<i>tnpR</i> <sup>mut135</sup>	<1	87	87	6	<0.05
VC0207	<i>res</i>	<i>tnpR</i> <sup>mut168</sup>	<0.1	8	80	8	0.2
VC0721	<i>res1</i>	<i>tnpR</i> <sup>mut135</sup>	<0.1	4	40	4	<0.05
VC1658	<i>res</i>	<i>tnpR</i> <sup>mut168</sup>	3	88	30	4	<0.05
VC2373	<i>res1</i>	<i>tnpR</i> <sup>mut168</sup>	0.1	3	30	4	0.1
VC0201	<i>res1</i>	<i>tnpR</i> <sup>mut135</sup>	0.6	17	28	4	<0.05
VCA0529	<i>res</i>	<i>tnpR</i>	0.2	2	20	4	<0.05
VC0874	<i>res1</i>	<i>tnpR</i>	<1	19	19	5	<0.05
VC1173	<i>res</i>	<i>tnpR</i> <sup>mut168</sup>	1	16	16	4	0.2
VC0202	<i>res1</i>	<i>tnpR</i>	7	94	13	3	<0.05
VC0203	<i>res1</i>	<i>tnpR</i> <sup>mut168</sup>	2	25	13	7	<0.05
VC1137	<i>res</i>	<i>tnpR</i> <sup>mut135</sup>	<1	12	12	8	<0.05
VC1535	<i>res</i>	<i>tnpR</i>	8	93	12	19	<0.05
VC1275	<i>res</i>	<i>tnpR</i>	8	85	11	4	<0.05
VC0130	<i>res1</i>	<i>tnpR</i> <sup>mut168</sup>	0.2	2	10	4	0.1
VC1338	<i>res1</i>	<i>tnpR</i>	3	30	10	4	0.05
VC2621	<i>res</i>	<i>tnpR</i> <sup>mut168</sup>	12	90	7.5	4	<0.05
VCA0632	<i>res1</i>	<i>tnpR</i>	11	77	7	3	<0.05
VC2419	<i>res</i>	<i>tnpR</i> <sup>mut135</sup>	5	34	6.8	4	<0.05
VC1500	<i>res</i>	<i>tnpR</i>	10	66	6.6	4	<0.05
VCA0752	<i>res</i>	<i>tnpR</i> <sup>mut168</sup>	4	24	6	7	<0.05
VCA0687	<i>res</i>	<i>tnpR</i> <sup>mut168</sup>	0.2	1	5	4	0.05
VC2705	<i>res</i>	<i>tnpR</i>	3	12	4	4	<0.05
VC1687	<i>res</i>	<i>tnpR</i> <sup>mut135</sup>	5	18	3.6	4	0.07
VC2742	<i>res1</i>	<i>tnpR</i> <sup>mut168</sup>	1.4	5	3.6	4	<0.05
VCA0016	<i>res1</i>	<i>tnpR</i> <sup>mut168</sup>	7	24	3.4	4	<0.05
VCA0242	<i>res1</i>	<i>tnpR</i>	2.5	8.4	3.4	4	0.1
VC2646	<i>res1</i>	<i>tnpR</i> <sup>mut135</sup>	8	18	2.2	3	0.07
VC2130	<i>res1</i>	<i>tnpR</i> <sup>mut168</sup>	35	76	2.2	4	<0.05
VC1619.1	<i>res</i>	<i>tnpR</i> <sup>mut168</sup>	13	25	1.9	8	<0.05
VCA0014	<i>res</i>	<i>tnpR</i> <sup>mut168</sup>	12	18	1.5	4	0.1
VC1034	<i>res1</i>	<i>tnpR</i>	75	100	1.3	4	<0.05

<sup>a</sup> Gene product designation by the Institute for Genomic Research.

<sup>b</sup> *res* or *res1* cassette in GOA1245 or GOA1246 strain background, respectively.

<sup>c</sup> Wild-type, mut168, or mut135 RBS mutant allele of *tnpR* gene fused to *V. cholerae* gene.

<sup>d</sup> Percent resolved CFU determined after 11 generations in LB broth.

<sup>e</sup> Percent resolved CFU determined after 24 h of infection in the infant mouse small intestine.

<sup>f</sup> The *ivi* genes were ranked by their ratio of percent resolution in vivo/in vitro.

<sup>g</sup> Number of mice used per experiment to determine percent resolution in vivo.

<sup>h</sup> Calculated by Student's two-tailed *t* test between the percent resolution values obtained in vivo and in vitro.

well as the absence of selective pressure placed upon bacteria during infection (22). However, the same sensitivity is also a major disadvantage in that many gene fusion strains that have low-to-moderate levels of transcription in vitro cannot be identified because such strains resolve and thus are eliminated during library construction. The screening method that we report here, which uses a combination of three *tnpR* alleles with different translation initiation efficiencies and two resolvable cassettes with different resolution efficiencies, minimizes this limitation. Our results show that this second-generation RIVET method is able to detect a wider range of *ivi* genes than the previous version (2).

Other improvements of our screening method include the incorporation of the *sacB* gene in the resolvable cassette and a preselection step to eliminate partially resolved strains. Livny and Friedman (13) recently reported a variation of RIVET, termed SIVET for "selectable IVET," in which resolution generates a functional chloramphenicol resistance gene, allowing positive selection of resolved strains. Inclusion of *sacB* in our resolvable cassette similarly allows positive selection of resolved strains (on agar supplemented with sucrose). The ability to positively select resolved strains and the preselection step have improved the efficiency and accuracy of the screening method. Indeed, the present screening method identified 85%

TABLE 4. Competition assays of *V. cholerae* *ivi* null mutant strains during growth in vitro and in the infant mouse small intestine

Gene product mutated	Function	Competitive index <sup>a</sup>	
		In vitro	In vivo
VC1500	Pqi family	3.9 <sup>b</sup>	0.74 <sup>b</sup>
VC2487	Conserved protein	0.56	0.049 <sup>b</sup>
VC0488	Extracellular binding protein	1.6 <sup>b</sup>	0.56
VC0622	Histidine kinase	2.2 <sup>b</sup>	0.61 <sup>b</sup>
VC0874	Hypothetical protein	0.95	0.045 <sup>b</sup>
VC0130	GGDEF protein	1.3	0.55
VC2705	Sodium symporter	0.57	0.07 <sup>b</sup>
VC2646	Phosphoenolpyruvate carboxylase	1.4 <sup>b</sup>	0.67 <sup>b</sup>

<sup>a</sup> Competitive indices were calculated as described in Materials and Methods. Each in vitro competition was done with two independent LB broth cultures, and each in vivo competition used between 8 and 10 infant mice.

<sup>b</sup> Significant difference from parent strain ( $P < 0.05$ ) by Student's two-tailed *t* test using as the control group competitions between parent strain GOA1264 and GOA6W, a virulent control strain harboring a plasmid insertion in *lacZ*.

of the putative *ivi* genes that were reconstructed and tested as bona fide *ivi* gene fusions whereas only 35% of those obtained by the prior RIVET screening method were *ivi* genes (2). In comparing our data with previous microarray data obtained from *V. cholerae* grown in LB broth, we see that the majority (98%) of our *ivi* genes fall below the 80th percentile of rank order gene expression in LB broth (24). This fact indicates that the preselection step that was added to the RIVET screening procedure was effective at removing fusion strains that are active in vitro.

Many *ivi* genes identified by this screening method could be important players in the adaptation of *V. cholerae* to the small intestine. The product of the VC1619.1 gene is similar to RTX toxins, which include cytolytic toxins (hemolysins and leukotoxins), metalloproteases, and lipases. The presence of tandemly repeated nonapeptides (GGXGXDX[L/I/V/W/Y/F]X; 6 to 40 times) is a sine qua non characteristic of this family of toxins. VC1619.1 has seven such repeats at its carboxy-terminal end. In addition, the protein has six cadherin-related domains in its central segment. This protein may contribute to induction of an inflammatory response in the infected host, as has been previously described for another *V. cholerae* toxin belonging to this family (6). Some of the *ivi* genes may play roles in adaptation to stressful conditions. VC0622 is homologous to BaeS (bacterial adaptive response), which has been shown to be involved in sensing cell envelope stresses, including spheroplast formation and overexpression of misfolded proteins (19). Also, the putative PqiA-homologous protein VC1500 may sense envelope stresses. This protein is an integral membrane protein and is related to proteins that are induced by the herbicide paraquat, which generates reactive oxygen species (9). Thioredoxin 2, which was also identified by our screening method, is known to play a role in oxidative stress responses in other bacteria (20). Another potentially important gene for host adaptation is the *mutT* ortholog VCA0632. Interestingly, *V. cholerae* has 11 paralogs of the MutT/nudix protein family. MutT-related proteins degrade 8-oxoguanosine, one of the most abundant kinds of DNA lesion resulting from oxidative stress (8). It is possible that prevention or correction of oxidative DNA damage is an important adaptive response of *V. cholerae* during infection (17).

Several of the *ivi* genes identified by this screening method are likely to be involved in nutrient acquisition. Two *ivi* genes related to phosphate acquisition (VC0721 and VC1687) were detected. In addition, one more phosphate-related gene was detected in the 94 putative *ivi* genes initially isolated (VC0723; polyphosphate kinase homolog). Also, two genes involved in glycogen metabolism (VCA0016 and VCA0014) were detected. The induction of these genes during infection suggests that in mice phosphate and energy sources are scarce and perhaps polyphosphates and stored carbohydrates are required for survival in this environment.

Approximately half (19 of 40) of the *ivi* gene products are predicted to be localized to the surface of *V. cholerae*. This indicates that genes detected by the RIVET system are enriched for those important for interaction with the surroundings, as would be expected from a theoretical point of view. On the other hand, of the 40 *ivi* genes identified only 1 may belong to a known class of virulence factors (putative RTX protein). This finding is not new (2) and suggests that the majority of the *ivi* genes may not belong to the standard virulence gene category.

In comparing the results of our screening method with those of a published microarray screening method (24), it should be noted that there is little overlap in the *ivi* genes detected. Only 15% of our genes (6 of 40) had levels of induction of  $>2$  in rabbit loop microarrays and, conversely, the microarray study identified approximately 700 genes with levels of induction of  $>2$  (ca. 22% of the total studied). Therefore, microarrays used to detect genes induced in vivo in animal tissues appear to be more sensitive and comprehensive than the RIVET method. One factor to consider in this light is that microarrays measure steady-state mRNA concentration changes rather than transcriptional initiation per se. Thus, if many of the genes that are induced in vivo are mostly regulated by mRNA degradation, the RIVET method may not be very suitable for their detection but microarrays would. On the other hand, microarrays can only be used to study gene expression at the time of sampling, whereas RIVET can detect genes that are turned on transiently at any stage of infection. Although additional microarray experiments can be done to examine expression at other times of infection, some times may not be feasible because not enough bacteria are present for isolation of sufficient amounts of RNA.

Genes VC1111 (*bioB*), VC1173 (*trpG*), and VCA0014 (*malQ*) and their respective metabolic pathways were previously reported to be essential for infectivity by signature-tagged mutagenesis screening methods (4, 14). In this and a related study (18), we found that 7 of the 10 *ivi* genes tested were important to achieve wild-type levels of intestinal colonization. Three of these mutants were only mildly attenuated, whereas four were attenuated  $>10$ -fold. This percentage (40%) is similar to the overall percentage (47%) of *ivi* genes that are required for virulence in animal models compiled from numerous IVET screening methods done on a number of different pathogens (1). Of the genes identified in this work, the most attenuated was VCA1008 (18). This result is interesting because VCA1008 was also the most highly induced *ivi* gene identified by our screening method. In a separate study, we have shown that VCA1008 is the only known porin in *V. cholerae* required for virulence (18). The next most attenuated



mutant strain (attenuated 20-fold) carried VC2487, which was also the second most highly induced *ivi* gene identified. However, after these two, this correlation between the level of transcriptional induction in vivo and importance for virulence no longer held, demonstrating that the tunable RIVET method is robust in finding bona fide virulence genes. A possible explanation for the lack of attenuation of VC0488 and VC0130 and the small attenuation levels of VC1500, VC0622, and VC2646 is redundancy. In the case of VC0130 (GGDEF protein), there are 40 other genes in *V. cholerae* that contain a GGDEF domain, each predicted to synthesize c-di-GMP. Consequently, a single mutation of only one gene of the entire family may not result in an observable phenotype.

Our results demonstrate that the second-generation RIVET method described herein is able to efficiently and reliably detect *V. cholerae* *ivi* genes in the infant-mouse model of cholera. The identification and characterization of such genes may contribute to the development of more efficacious vaccines to protect against cholera.

#### ACKNOWLEDGMENTS

We thank members of the Center for Vaccine Development in Baltimore for generous help in preparing the gene fusion library.

This work was supported by Pew Latin American Fellowship P0337SC to C.G.O and NIH grants AI45746 and AI55058 to A.C. and AI19716 to J.B.K.

#### REFERENCES

1. Angelichio, M. J., and A. Camilli. 2002. In vivo expression technology. *Infect. Immun.* **70**:6518–6523.
2. Camilli, A., and J. J. Mekalanos. 1995. Use of recombinase gene fusions to identify *Vibrio cholerae* genes induced during infection. *Mol. Microbiol.* **18**:671–683.
3. Chiang, S. L., and J. J. Mekalanos. 1998. Use of signature-tagged transposon mutagenesis to identify *Vibrio cholerae* genes critical for colonization. *Mol. Microbiol.* **27**:797–805.
4. Chiang, S. L., J. J. Mekalanos, and D. W. Holden. 1999. In vivo genetic analysis of bacterial virulence. *Annu. Rev. Microbiol.* **53**:129–154.
5. Donnenberg, M. S., and J. B. Kaper. 1991. Construction of an *eae* deletion mutant of enteropathogenic *Escherichia coli* by using a positive-selection suicide vector. *Infect. Immun.* **59**:4310–4317.
6. Fullner, K. J., J. C. Boucher, M. A. Hanes, G. K. Haines III, B. M. Meehan, C. Walchle, P. J. Sansonetti, and J. J. Mekalanos. 2002. The contribution of accessory toxins of *Vibrio cholerae* O1 El Tor to the proinflammatory response in a murine pulmonary cholera model. *J. Exp. Med.* **195**:1455–1462.
7. Heidelberg, J. F., J. A. Eisen, W. C. Nelson, R. A. Clayton, M. L. Gwinn, R. J. Dodson, D. H. Haft, E. K. Hickey, J. D. Peterson, L. Umayam, S. R. Gill, K. E. Nelson, T. D. Read, H. Tettelin, D. Richardson, M. D. Ermolaeva, J. Vamathevan, S. Bass, H. Qin, I. Dragoi, P. Sellers, L. McDonald, T. Utterback, R. D. Fleishmann, W. C. Nierman, and O. White. 2000. DNA sequence of both chromosomes of the cholera pathogen *Vibrio cholerae*. *Nature* **406**:477–483.
8. Imlay, J. A., S. M. Chin, and S. Linn. 1988. Toxic DNA damage by hydrogen peroxide through the Fenton reaction in vivo and in vitro. *Science* **240**:640–642.
9. Koh, Y. S., and J. H. Roe. 1995. Isolation of a novel paraquat-inducible (*pqi*) gene regulated by the *soxRS* locus in *Escherichia coli*. *J. Bacteriol.* **177**:2673–2678.
10. Kolter, R., M. Inuzuka, and D. R. Helinski. 1978. Trans-complementation-dependent replication of a low molecular weight origin fragment from plasmid R6K. *Cell* **15**:1199–1208.
11. Lally, E. T., R. B. Hill, I. R. Kieba, and J. Korostoff. 1999. The interaction between RTX toxins and target cells. *Trends Microbiol.* **7**:356–361.
12. Lee, S. H., D. L. Hava, M. K. Waldor, and A. Camilli. 1999. Regulation and temporal expression patterns of *Vibrio cholerae* virulence genes during infection. *Cell* **99**:625–634.
13. Livny, J., and D. I. Friedman. 2004. Characterizing spontaneous induction of Stx encoding phages using a selectable reporter system. *Mol. Microbiol.* **51**:1691–1704.
14. Merrell, D. S., D. L. Hava, and A. Camilli. 2002. Identification of novel factors involved in colonization and acid tolerance of *Vibrio cholerae*. *Mol. Microbiol.* **43**:1471–1491.
15. Michalski, J., J. E. Galen, A. Fasano, and J. B. Kaper. 1993. CVD110, an attenuated *Vibrio cholerae* O1 El Tor live oral vaccine strain. *Infect. Immun.* **61**:4462–4468.
16. Miller, V. L., and J. J. Mekalanos. 1988. A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *toxR*. *J. Bacteriol.* **170**:2575–2583.
17. O'Rourke, E. J., C. Chevalier, A. V. Pinto, J. M. Thiberge, L. Ielpi, A. Labigne, and J. P. Radicella. 2003. Pathogen DNA as target for host-generated oxidative stress: role for repair of bacterial DNA damage in *Helicobacter pylori* colonization. *Proc. Natl. Acad. Sci. USA* **100**:2789–2794.
18. Osorio, C. G., H. Martinez-Wilson, and A. Camilli. 2004. The *ompU* paralogue *vca1008* is required for virulence of *Vibrio cholerae*. *J. Bacteriol.* **186**:5167–5171.
19. Raffa, R. G., and T. L. Raivio. 2002. A third envelope stress signal transduction pathway in *Escherichia coli*. *Mol. Microbiol.* **45**:1599–1611.
20. Raman, S., T. Song, X. Puyang, S. Bardarov, W. R. Jacobs, Jr., and R. N. Husson. 2001. The alternative sigma factor SigH regulates major components of oxidative and heat stress responses in *Mycobacterium tuberculosis*. *J. Bacteriol.* **183**:6119–6125.
- 20a. Reed, R. R. 1981. Transposon-mediated site-specific recombination: a defined in vitro system. *Cell* **25**:213–219.
21. Senanayake, S. D., and D. A. Brian. 1995. Precise large deletions by the PCR-based overlap extension method. *Mol. Biotechnol.* **4**:13–15.
22. Schlauch, J. M., and A. Camilli. 2000. IVET and RIVET: use of gene fusions to identify bacterial virulence factors specifically induced in host tissues. *Methods Enzymol.* **326**:73–96.
23. Tischler, A. D., and A. Camilli. 2004. Cyclic diguanylate (c-di-GMP) regulates *Vibrio cholerae* biofilm formation. *Mol. Microbiol.* **53**:857–869.
- 23a. Tischler, A. D., S. H. Lee, and A. Camilli. 2002. The *Vibrio cholerae* *vieSAB* locus encodes a pathway contributing to cholera toxin production. *J. Bacteriol.* **184**:4104–4113.
24. Xu, Q., M. Dziejman, and J. J. Mekalanos. 2003. Determination of the transcriptome of *Vibrio cholerae* during intractintestinal growth and midexponential phase in vitro. *Proc. Natl. Acad. Sci. USA* **100**:1286–1291.