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Use of recombinase gene fusions to identify *Vibrio cholerae* genes induced during infection

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Summary

A complete understanding of host-parasite interactions must necessarily include the identification and characterization of gene products expressed by both parties during the infectious process. We have developed a new screen to identify bacterial genes that are transcriptionally induced during infection of a host animal. The method is based on pre-selection of strains carrying *tnpR* operon fusions (encoding resolvase, a site-specific DNA recombinase) which are not expressed *in vitro,* followed by screening for a subset of these strains that subsequently express resolvase within the host environment. The latter subset was recognized as recombinants that had deleted a resolvase-specific reporter construct. Thirteen transcription units of *Vibrio cholerae* were identified that were induced during infection in an infant mouse model of cholera. Five of these were predicted to encode polypeptides with diverse functions in metabolism, biosynthesis and motility; one encoded a secreted lipase; two appear to be antisense to genes involved in motility; and five are predicted to encode polypeptides of unknown function. Three of the transcripts were shown to be required for full virulence in infant mice, as assessed by competition experiments.

Introduction

Vibrio cholerae is a Gram-negative bacterium responsible for severe epidemic and endemic diarrhoea in many parts of the world (Wachsmuth *et al.,* 1994). The profuse watery diarrhoea, which serves to spread *V. cholerae* into the environment and eventually to other human hosts, is caused, in large part, by the action of cholera toxin (Ctx) on the small intestinal epithelium (Gill, 1977). Several other virulence factors of this pathogen, such as the major colonizing factor TCP pili have been identified in the laboratory by virtue of their co-ordinate regulation with Ctx (Peterson and Mekalanos, 1988; Taylor *et al.,* 1987). However, given the complexity of the host gastrointestinal tract, it is likely that there are additional virulence factors which are not co-regulated with Ctx *in vitro.* It is further likely that a multitude of other bacterial factors which facilitate survival, growth and subsequent spread from the host have escaped our detection because the *in vivo* conditions required to induce their expression have not been duplicated in the laboratory.

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Several experimental approaches have recently been reported that have allowed identification of genes that are expressed by pathogens within host animals or cells. Mahan *et al.* (1993) recently reported the development of *in vivo* expression technology (IVET), a method that allows the direct selection of bacterial gene fusions expressed in the host compartment via complementation of an attenuating auxotrophic mutation. Subsequent *in vitro* screening of a co-expressed *lacZY* operon fusion allowed the identification of gene fusions that were not expressed during growth on laboratory media. Gene fusions to an antibiotic resistance and other selectable genes have also recently been used to select directly for gene fusions expressed in host animals (Mahan *et al.,* 1995) and tissue culture (Berger and Isberg, 1993; Klarsfeld *et al.,* 1994). Finally, subtractive hybridization has been used to identify *in vivo*-induced transcripts (Plum and Clark-Curtiss, 1994). However, the short half life of bacterial mRNA and the difficulty in obtaining sufficient numbers of bacterial cells from infected tissues has so far limited this last approach to tissue culture models. A limitation of all these approaches is that they inherently select more strongly for those genes that are continuously and highly expressed within the host. *In vivo*-expressed genes that display transient bursts of expression would not be expected to be revealed by any of these approaches. Accordingly, we have designed a genetic ‘screen’ to identify bacterial genes that are transcriptionally silent during growth in the laboratory but which are then transiently or constitutively induced within the host to either low or high levels of transcription. Our approach is based on the recently developed method of assaying gene expression via transcriptional fusions to the site-specific recombinase resolvase (Camilli *et al.,* 1994). In this report, we describe the application of this approach to identify genes of *V. cholerae* which are transcriptionally induced in an infant mouse model of cholera (Wachsmuth *et al.,* 1994).

Results

Construction of *V. cholerae* transcriptional fusion libraries

Plasmid pIVET5 was constructed to facilitate the generation of random *V. cholerae* transcriptional fusions to a promoterless synthetic operon consisting of *tnpR* and *lacZY* reporter genes (Fig. 1). *tnpR* codes for the site-specific recombinase resolvase from Tnγδ (Reed, 1981). Random fragments of genomic DNA from both *V. cholerae* classical and El Tor biotypes were cloned immediately in front of the synthetic operon and the resulting recombinant plasmid libraries were transferred into classical and El Tor reporter strains, respectively; whereupon the non-replicating plasmids integrated into the genomes by homologous recombination. As illustrated in Fig. 1, each resulting integration strain contained a duplication of the cloned putative promoter-containing *V. cholerae* sequence such that each strain remained wild type with respect to expression of the downstream gene or operon. The classical and El Tor reporter strains used in these library constructions contained the resolvase substrate sequences *res1–tet–res1* and *res–tet–res* on their respective chromosomes within the *lacZ* locus (see the *Experimental procedures),* which resulted in $LacZ^{-}$ and tetracyline-resistant (Tc^{R}) backgrounds. Note that the *res1–tet–res1* and *res–tet–res* sequences are unlinked to the integrated *tnpR* fusions in each strain, in contrast to the system previously described by Camilli *et al.* (1994). The mutant *res1* sequences contained a T to C transition at their crossover sites resulting in approximately 10-fold decreased

recombination efficiency (Newman and Grindley, 1984). This allowed us to examine two levels of gene expression in the different *V. cholerae* biotypes, whereby the El Tor biotype reporter strain was more sensitive to low levels of *tnpR* expression than the classical biotype reporter strain. Expression of resolvase in each strain background catalysed excision of *tet* out of the bacterial chromosome (Camilli *et al.,* 1994). The excised *tet* gene is present on a non-replicating DNA circle which is segregated by cell division leading to a Tc^S phenotype in descendent bacteria (Camilli *et al.,* 1994).

Screening for *V. cholerae* transcripts induced *in vivo*

When each transcriptional fusion library was plated onto a rich agar media containing Xgal, approximately 40% of the resulting colonies were phenotypically $LacZ^-$ and, therefore, were predicted to have transcriptionally inactive fusions to the synthetic operon. Pools of 200 $LacZ^-$ Tc^R strains were introduced individually into single CD-1 suckling mice. After 24 h, bacteria were recovered from homogenates of upper intestinal tissue by plating on Luria–Bertani (LB) agar containing streptomycin. Replica plating was then used to identify those *in vivo* recovered clones that were Tc^S (Fig. 1). Of approximately 13000 $LacZ^-$ and Tc^R strains screened, 56 Tc^S strains were recovered from the infant mice small intestines after 24h. Therefore, approximately 0.1–0.2% of strains from the original library contained *in vivo*-induced transcriptional fusions to the *tnpR–lacZY* synthetic operon. In control experiments in which the parental strain AC-V66 ($TnpR^-$) was used to infect infant mice, no Tc^S bacteria were recovered. Therefore, the *res–tet–res* reporter substrate for TnpR was completely stable during infection in the absence of *tnpR* fusions.

Confirmation of *in vivo* induction of *V. cholerae* transcriptional fusions

From each of the 56 Tc^S strains isolated above, the integrated plasmids containing the synthetic operon fusions were recovered into *Escherichia coli* (see the *Experimental procedures*) for subsequent analysis and to allow the reconstruction of each fusion in a fresh reporter strain background. Restriction enzyme analysis of each of the 56 plasmids enabled us to eliminate those having identical *V. cholerae* DNA inserts (data not shown), resulting in 37 plasmids that were unique. In all cases of identical plasmid inserts, their parent strains were found to have originated from the same pool and were, therefore, considered to have been siblings.

Each of the 37 unique plasmids was integrated into the chromosome of their respective parental strain to regenerate each transcriptional fusion in a fresh *res–tet–res* (El Tor) or *res1–tet–res1* (classical) background. This allowed us to independently test each strain for its resolution levels when grown *in vitro* and during infection of infant mice. Of the 37 reconstructed fusion strains examined, 13 reproducibly resolved to a greater extent during infection of infant mice than when grown *in vitro* (Table 1). Of the remaining 24 strains, most had very low resolution levels when grown either *in vitro* or in infant mice (data not shown) and, therefore, apparently arose as a result of low level constitutive resolution events that occurred during the original mouse infections. These 24 strains were considered ‘background’ inherent in our screening protocol and were not analysed further.

None of the *tnpR–lacZY* fusions to putative *in vivo*-induced genes were inducible by growth of the strains in a low-iron medium, under microaerophilic conditions in a rich medium or when grown under *in vitro* conditions (Iwanga *et al.,* 1986; Miller and Mekalanos, 1988), which maximally induce expression of the ToxR regulon in *V. cholerae* classical and El Tor biotypes (data not shown). Given that the ToxR regulon includes many of the known virulence determinants of *V. cholerae,* these data suggested that the TnpR fusion approach was apparently identifying new classes of potential virulence genes.

A DNA sequencing primer designed to read out from the N-terminal coding end of *tnpR* allowed us to determine the sequence of approximately 300 bases of *V. cholerae* DNA fused to the synthetic operon in the 13 strains shown in Table 1. In 11 of the strains, the synthetic operon was found to be transcriptional fused within a predicted open reading frame (ORF) based on preferred codon usage (data not shown) and, in the majority of cases, on extensive similarity of the predicted amino acid sequences to known bacterial proteins (see below). The remaining two strains contained fusions of the synthetic operon to putative anti-sense transcripts.

In vivo-induced loci involved in amino acid biosynthesis and general metabolism

Strain AC-V226 contained a synthetic operon fusion within the N-terminal end of a predicted ORF, designated *iviI* (*in vivo*-induced transcript I) (Fig. 2). The deduced amino acid sequence of *ivil* displayed a high degree of similarity to the *Salmonella typhimurium* Cysl protein (J. Ostrowski, J. Y. Wu, D. C. Rueger, B. E. Miller, L. M. Siegel, and N. M. Kredich, unpublished data), which catalyses an intermediate step in the biosythesis of L-cysteine from inorganic sulphate (Siegel *et al.,* 1973). Evidence of an upstream ORF, *orfU*, encoding a homologue of the *S. typhimurium* CysJ protein was also obtained (data not shown). Therefore, *V. cholerae* appears to have maintained the order of at least the first two genes of the *cysJIH* operon as found in *S. typhimurium* (Jones-Mortimer, 1973). Beta-galactosidase activity was assayed for strain AC-V226 (*iviI: :tnpR–lacZY*) and was found to be induced 21-fold in a minimal medium lacking L-cysteine (667 versus 32 units of activity during growth in media without or with L-cysteine, respectively). An *iviI* (*cysI*) mutant strain of *V. cholerae,* AC-V172 (see below), was accordingly found to be auxotrophic for L-cysteine.

Strain AC-V228 contained a fusion within the middle of *iviIII* (Fig. 2) which was predicted to encode a polypeptide that had high similarity to the *Escherichia coli* ArgA protein. ArgA catalyses the first step in the biosynthesis of L-arginine (Brown *et al.,* 1987). Beta-galactosidase activity was assayed for strain AC-V228 (*iviIII: :tnpR–lacZY)* and was found to be induced 3.5-fold in a minimal medium lacking L-arginine (98 versus 28 units of activity during growth in media without or with L-arginine, respectively). An *iviIII* (*argA*) mutant strain of *V. cholerae,* AC-V176 (see below), was accordingly found to be auxotrophic for L-arginine.

Strain AC-V227 contained a fusion within the middle of *iviII* (Fig. 2) which was predicted to encode a polypeptide that had high similarity to the *E. coil* SucA protein (Darlison *et al.,* 1984). SucA is part of a multisubunit enzyme that catalyses a step in the tricarboxylic acid (TCA) cycle whereby α-ketoglutarate is oxidatively decarboxylated to succinyl CoA and

carbon dioxide. We were unable to modulate β-galactosidase activity from this strain by growth in conditions known to induce transcription of *sucA* in *E. coli* (Nimmo, 1987) (data not shown).

Strain AC-V230 contained a fusion within the C-terminal end of *iviV* (Fig. 2) which was predicted to encode a polypeptide that had high similarity to the *Pseudomonas stutzeri* NirT protein, *nirT* codes for a tetrahaem protein of unknown function; the gene is located within a nitrite reduction operon (*nirSTBM*) and its product is required for respiratory nitrate reduction (Zumft *et al.,* 1988). Expression of *nirT* in *P. stutzeri* was hypothesized to be induced by anaerobic growth in the presence of nitrate and/or nitrite (Juengst *et al.,* 1991; Liu *et al.,* 1983). However, we were unable to induce expression of the *iviV::tnpR–lacZY* fusion in *V. cholerae* under similar growth conditions, as measured by β-galactosidase activity (data not shown).

In vivo-induced loci involved in motility and chemotaxis

Strain AC-V229 contained a fusion within the middle of *iviIV* (Fig. 3) which was predicted to encode a polypeptide that had similarity to members of the bacterial methyl-accepting chemoreceptor protein family. A moderate level of *iviIV* transcription during *in vitro* growth was suggested by the resolution data in Table 1.

Strain AC-V233 contained a fusion within the non-coding 3′ end of an RNA transcript containing *orf4*; this was predicted to encode a polypeptide that was a flagellin subunit protein (Fig. 3). However, the fusion junction occurred in the opposite transcriptional orientation to and between the predicted stop codon and transcriptional terminator for *orf4.* This suggested that the *tnpR* fusion occurred within an RNA transcript induced during infection which was anti-sense to at least the non-coding end of the *orf4* RNA transcript. Interestingly, a downstream ORF, *orf5*, also was predicted to encode a polypeptide that had high similarity to other flagellin subunit proteins. A putative σ^{28}-dependent promoter was located immediately upstream of *orf5* (data not shown). Therefore, *V. cholerae* may express multiple flagellin subunit proteins, as do many other motile Gram-negative bacterial species (Nuijten *et al.,* 1990; Pleier and Schmitt, 1989).

Strain AC-V234 contained a fusion within the C-terminal end of *orf3* (Fig. 3) which was predicted to encode a polypeptide that had similarity to the *Bacillus subtilis* CheV protein (Fredrick and Helmann, 1994). The *B. subtilis* CheV protein is a novel ‘chimaeric’ protein which has CheY and CheW homologous domains and was shown to be required for normal chemotaxis (Fredrick and Helmann, 1994). However, the *tnpR* fusion in AC-V234 occurred in the opposite transcriptional orientation to that predicted for *orf3.* This suggested that the synthetic operon fusion occurred within an RNA transcript induced during infection which was anti-sense to the C-terminal coding end of *orf3.* This putative RNA transcript may have originated from an upstream ORF, *orf2,* which is in the proper transcriptional orientation to induce the synthetic operon (Fig. 3). However, *orf2* had a predicted transcriptional terminator at its 3′ end which may prevent transcription into the downstream *orf3* region, *orf2* was predicted to encode a polypeptide that had similarity to the *Erwinia chrysanthemi* PecS regulatory protein (data not shown) which regulates expression of several proteins including the secreted pectinase and cellulase enzymes (Reverchon *et al.,* 1994).

In vivo-induced locus encoding a secreted lipase

Strain AC-V237 contained a fusion within the middle of *iviXII* which was identical in DNA sequence to a partially sequenced ORF designated the *hlyC* gene of *V. cholerae* (Casanova and Peterson, 1995; Manning *et al.,* 1984). *hlyC* is an uncharacterized gene which was predicted to encode a secreted triacylglyceride-specific lipase, based on similarity to other bacterial lipases. Our analysis confirmed this observation by finding similarity of the predicted HlyC (IviXII) polypeptide to the *Pseudomonas aeruginosa* LipA protein (Fig. 4), including the heptapeptide consensus motif (IGHSHGG) thought to lie at the active site of this family of lipases (Wohlfarth *et al.,* 1992) (data not shown). A *hlyC* (*iviXII*)-containing mutant strain of *V. cholerae* that we constructed, AC-V195 (see below), lost the ability to produce a clearing zone around colonies grown for 48 h on agar media containing emulsified tributyrin (Jorgensen *et al.,* 1991) (Fig. 5), thus demonstrating that the *htyC* (*iviXII*) locus is required for this secreted lipase activity.

In vivo-induced loci encoding polypeptides of unknown function

Strain AC-V231, which showed the highest level of *in vivo*-induction (Table 1), contained a fusion within the middle of *iviVI* (Fig. 6) which was predicted to encode a polypeptide that had high similarity to members of the ATP-binding cassette family of transporters. The sequenced portion of *iviVI* included an N-terminal ATP-binding Walker A box motif (GPS/TGXGKS/T), which is highly conserved in members of this transporter family (Ames *et al.,* 1990). The best similarity obtained was to the *Synechococcus* PCC 7942 CysA protein, which is part of a three-component transporter for sulphate (Green *et al.,* 1989). Accordingly, β-galactosidase activities and resolution were assayed for strain AC-V231 (*iviVI::tnpR–lacZY*) after growth in a minimal medium lacking L-cysteine and containing sulphate as the sole source of sulphur. However, the AC-V231 gene fusion was not induced in this medium. In addition, an *iviVI*-containing mutant strain of *V. cholerae*, AC-V184 (see below), was not auxotrophic for L-cysteine in a minimal medium containing sulphate as the sole source of sulphur. Therefore, *iviVI* appeared not to encode the sole *V. cholerae* transporter of sulphate.

Strain AC-V232 contained a fusion within the N-terminal end of *iviVII* (Fig. 6B) whose predicted polypeptide sequence had no significant similarity to any sequences in the searched databases. Evidence of a downstream ORF, *orf1*, was also obtained, which was predicted to encode a polypeptide that had high similarity to the *Vibrio alginolyticus* collagenase protein (Takeuchi *et al.,* 1992). It is not known at this time whether *iviVII* and *orf1* are co-regulated and/or are functionally related in any way.

Strain AC-V236 contained a fusion within the middle of *iviXI* (Fig. 6) which was predicted to encode a polypeptide with high similarity to a hypothetical 21.7 kDa *E. coli* polypeptide of unknown function. AC-V236 exhibited a moderate level of resolution during *in vitro* growth but resolved to a higher level in infant mice (Table 1).

Strains AC-V235 and AC-V238 contained fusions within *iviX* and the C-terminal end of *iviXIII*, respectively, whose predicted polypeptide sequences had no significant similarities to any sequences in the searched databases.

Competition assays of *V. cholerae* mutant strains in infant mice

To test whether any of the *in vivo*-induced and associated loci discussed above contributed to colonization and growth in infant mice, we examined strains mutated in each locus in an infant mouse competition assay. Each gene was mutated by single integration of the non-replicating plasmid pGP704, with the exception of *iviII* for which we repeatedly failed to obtain an integration, suggesting that insertional inactivation of the *V. cholerae sucA* homologue was lethal for growth on standard laboratory media. Each mutant strain was competed against its isogenic, virulent parent strain, which was phenotypically $LacZ^{-}$ as a result of integration of pGP704 into the endogenous *V. cholerae lacZ* gene. The majority of mutant strains had competitive indices of approximately 1 (Table 2) and, therefore, exhibited no colonization defects in this competition assay. However, mutant strains AC-V176 (*iviIII* (*argA*)), AC-V186 (*iviVII*) and AC-V196 (*iviXIII*) reproducibly exhibited small colonization defects in two independent experiments and their numbers were reduced by five-, two- and threefold, respectively, compared with the wild-type strain. Interestingly, the mutant strain AC-V189 (*orf3* (CheV similarity)) colonized at least twofold better than the wild type in two independent experiments. This mutant exhibited a hyperswarming phenotype when grown in semi-solid agar, consistent with a possible role for *orf3* in the chemotaxis phenotype of *V. cholerae*.

Discussion

The data presented here demonstrate the utility of using a gene encoding a site-specific DNA recombinase as a reporter to identify those RNA transcripts of a pathogenic bacterium which are induced during infection of a host animal. Thirteen transcription units of *V. cholerae* were identified which were induced after intragastric inoculation of infant mice. Five of these are predicted to encode polypeptides that could be linked with diverse functions in metabolism, biosynthesis and motility; one was similar to a secreted lipase; five are predicted to encode polypeptides of unknown function in that they either failed to exhibit any significant similarity to protein sequences in the databases or had similarity to proteins of unknown function; and, two may be antisense RNA transcripts, both of which lie within or near genes important for bacterial motility. Three of the thirteen *in vivo*-induced transcripts identified in this study contributed to the virulence of *V. cholerae* in the infant mouse model of cholera. This fulfilled our expectation that the identification of genes specifically induced during infection will yield an enrichment for genes which contribute to colonization, survival and/or growth within the host.

In vivo-induced transcripts involved in biosynthesis and metabolism

Two *in vivo*-induced amino acid biosynthetic genes were identified, including one for L-cysteine and for L-arginine. A mutant strain for the latter gene was attenuated fivefold in competition assays, suggesting that L-arginine was limiting for bacterial growth in the infant mouse small intestine. Klarsfeld *et al.* (1994) recently reported that the *Listeria monocytogenes arpJ* gene, encoding a subunit of an L-arginine transporter, was transcriptionally induced during infection of a tissue-culture cell line. Perhaps free L-arginine, which is an essential amino acid supplied only through the diet, is in low concentration in host tissues, or, alternatively, bacteria have an increased demand for

arginine *in vivo*. The arginine biosynthetic pathway feeds into the pathway for polyamines (Glansdorff, 1987). It is possible that increased *iviII* (*argA*) transcription occurs in response to decreased intracellular arginine pools that result from utilization of arginine for polyamine biosynthesis. Perhaps bacterial polyamines play undefined roles in the virulence of a variety of bacterial pathogens.

A mutant strain for the L-cysteine biosynthetic gene *iviI* (*cysI*) appeared fully virulent in infant mouse competition assays. Adult rats have been found to maintain high concentrations of cysteine in their small intestinal mucosa and lumen regardless of diet or short-term starvation (Dahm and Jones, 1994). However, cysteine concentrations in the infant mouse small intestine are not known. Therefore, it is possible that the concentrations of available L-cysteine in the infant mouse small intestine may have been limiting enough to induce transcription of the *V. cholerae iviI* (*cysI*) gene, yet not limiting for bacterial growth. Alternatively, if available L-cysteine was limited, it is possible that the *iviI* (*cysI*) mutation, which would block reduction of sulphite to sulphide in the L-cysteine biosynthetic pathway, may have been bypassed by available sulphide and/or thiosulphate within the infant mouse small intestine.

Interestingly, a second gene predicted to be involved in sulphate metabolism was identified in this study, *iviVI* is predicted to code for an ATP-binding cassette transporter with similarity to the sulphate uptake transporter CysA of *Synechococcus* PCC 7942 (Green *et al.,* 1989). However, transcription of *iviVI* was not inducible by growth of *V. cholerae* AC-V231 under conditions of L-cysteine limitation with sulphate present as the sole source of sulphur. Furthermore, an *iviVI* mutant strain of *V. cholerae* was competent for growth in a minimal medium containing sulphate as the sole source of sulphur. Therefore, it is unlikely that *iviVI* codes for a sulphate transporter, *iviVI* was the most highly *in vivo*-induced gene found in this study yet was not inducible during growth in the laboratory under microaerophilic conditions, in a minimal medium, a low-iron medium or in a medium known to maximally induce expression of the ToxR virulence gene regulon. Although an *iviVI* mutant strain of *V. cholerae* was not attenuated in infant mouse competition experiments, it would be of interest to understand the regulation of this gene, which appears to respond specifically to signals which occur in the host.

The *in vivo*-induced metabolic genes identified included one encoding a TCA cycle enzyme subunit and one encoding a tetrahaem protein presumably involved in nitrite reduction. In *E. coli,* the gene for the former, *sucA,* is induced by aerobic growth on acetate and repressed by growth anaerobically or in the presence of high glucose concentrations (Nimmo, 1987). We were unable to alter expression of the *V. cholerae iviII* (*sucA*) gene under similar growth conditions, which suggested differing regulation to that in *E. coli*. A *V. cholerae* mutant strain for *iviII* was not obtained, presumably because of lethality, at least under standard conditions of laboratory growth.

The *P. stutzeri nirT* gene codes for a tetrahaem polypeptide that is required for anaerobic respiratory nitrate reduction and whose expression is presumably induced by anaerobic conditions (Juengst *et al.,* 1991; Liu *et al.,* 1983). A mutant strain for the *V. cholerae nirT* homologue (*iviV*) appeared fully virulent in the infant mouse competition assay. Therefore,

either *V. cholerae* has a redundant mechanism for reducing nitrite or, alternatively, reduction of nitrite is not required for survival and/or growth in the infant mouse assay. Oxygen tension in the adult rat (both germ-free and conventional) intestinal lumen and at the mucosal surface is low (Bornside *et al.,* 1976). Growth of *V. cholerae* in the infant mouse intestinal lumen is believed to reduce the presumed low oxygen tension in this compartment further (Freter *et al.,* 1981). We could not, however, show that the *V. cholerae nirT* homologue (*iviV*) was induced by anaerobic growth conditions *in vitro.* It is, therefore, possible that *iviV* may represent one member of a regulon that is induced by another host signal(s) that acts as a surrogate signal for the low oxygen tension in the intestinal lumen.

In vivo-induced transcripts involved in motility and chemotaxis

Three of the *in vivo*-induced transcription units identified in this study relate to bacterial motility and chemotaxis. There is evidence that suggests that motility and chemotaxis are required for full virulence of *V. cholerae* in various adult animal models of cholera (Freter *et al.,* 1981; Richardson, 1991). In contrast, in the infant mouse model of cholera, chemotactic motility is actually a detriment in that non-chemotactic mutant strains survive in greater numbers than wild-type vibrios and produce a more rapid and severe disease (Freter and O'Brien, 1981). One of the *in vivo*-induced transcripts we found, *iviIV,* was predicted to encode a methyl-accepting chemoreceptor and was shown to be transcribed at significant levels both *in vitro* and *in vivo,* as measured by resolution levels. Expression of *MIV* appeared to be independent of ToxR regulation, in contrast to the recently described *acfB* and *tcpI* genes which encode putative methyl-accepting chemoreceptor proteins and which are positively regulated by ToxR (Everiss *et al.,* 1994; Harkey *et al.,* 1994). Strains mutated in *acfB* and *tcpI* exhibited altered chemotaxis *in vitro* (Everiss *et al.,* 1994; Harkey *et al.,* 1994) but their contribution to colonizing the small intestine, if any, is not known at present. A mutant strain for *iviIV* exhibited normal chemotaxis *in vitro* and appeared fully virulent in the infant mouse competition assay. Therefore, chemotaxis mediated by this protein was neither required for nor a detriment to colonization of the infant mouse.

Another *in vivo*-induced fusion (*iviIX*) was found to be anti-sense to *orf3*, which encoded a protein with similarity to CheV of *B. subtilis.* An *orf3* mutant strain of *V. cholerae* exhibited a hyperswarming phenotype *in vitro* and appeared to outcompete the wild-type strain in infant mice. Therefore, chemotaxis mediated by Orf3 was a detriment to wild-type vibrios; this is in agreement with the observations of Freter and O'Brien (1981). A second *in vivo*-induced fusion (*iviVIII*) was also found to be antisense, in this case to the non-coding sequence 3′ of *orf4* which is predicted to encode a flagellin protein. Any biological significance for these anti-sense transcripts is not known at present. However, it is tempting to speculate that they are either directly involved in down-regulating *orf3* and *orf4* expression or are artifactual in nature but reflect reduced levels of *orf3* and *orf4* transcription at some stage of infection. Recent evidence indicates that motility and ToxR-activated gene expression are reciprocally regulated, suggesting that at a stage in the infection cycle where cholera toxin and TCP pili are optimally expressed, motility gene expression may be repressed (Gardel and Mekalanos, 1994; Harkey *et al.,* 1994). Our identification of various motility and chemotaxis genes and associated RNA transcripts should aid further study to

assess the role(s) of these bacterial properties in the virulence and pathogenicity of *V. cholerae*.

In vivo-induced transcript coding for a secreted lipase

One *in vivo-induced* gene found in this study was identical to the previously sequenced *hlyC* gene of *V. cholerae,* which was predicted (Casanova and Peterson, 1995; Manning *et al.,* 1984) and demonstrated here to encode a secreted lipase, *hlyC* lies downstream from the *hlyAB* genes, which encode a secreted haemolysin and a methyl-accepting chemoreceptor homologue, respectively (Aim and Manning, 1990; Jeffery and Koshland, 1993; Manning *et al.,* 1984). Although by examination of their nucleotide sequence, the *hlyA, hlyB* and *hlyC* genes do not appear to comprise a classical operon, it is possible they are co-regulated at least in part by HlyU, a recently described transcriptional activator of *hlyA* (Williams *et al.,* 1993). A strain containing the *hlyU* mutation was recently shown to be attenuated for virulence in the infant mouse model and furthermore, the observed attenuation was not fully explained by loss of HlyA expression (Williams *et al.,* 1993). Therefore, HlyU may regulate additional virulence factors and, if it is responsible for expression of *hlyC*, it follows that HlyU may be a regulator that responds to *in vivo* environmental signals. Stoebner and Payne (1988) have reported an increase in haemolysin (*hlyA*) expression under low iron conditions. However, we did not observe induction of our *hlyC*::*lacZ* fusion by growth in media containing low iron levels. Therefore, at one level at least (i.e. low iron induction), *hlyA* and *hlyC* appear to be regulated independently.

A *hlyC* mutant strain appeared fully virulent in the infant mouse competition assay. However, it is possible that the co-inoculated wild-type strain was able to complement a lipase defect of the mutant strain *in trans.* Roles for the haemolysin and lipase in virulence and/or pathogenesis are not known, but it is possible that they act within the host small intestine, serving perhaps a defensive and/or nutritional role, for example, by damaging host immune cells by hydrolysing membrane lipids and thus releasing fatty acids; *V. cholerae* could potentially metabolize the released fatty acids. Mahan *et al.* (1995) have recently described an *in vivo*-induced *S. typhimurium* gene, *fadB*, which codes for a subunit of a multienzyme complex capable of degrading long-chain fatty acids. Therefore, metabolism of host lipids may be a common activity of bacterial pathogens.

In vivo-induced transcripts are not inducible *in vitro* under various growth conditions

None of the *in vivo*-induced *V. cholerae* transcription units identified in this study was regulated *in vitro* by conditions in which iron was limiting for growth, under microaerophilic growth conditions or by growth conditions which modulate members of the ToxR virulence gene regulon. The last result was not unexpected because, in preliminary experiments, we found that ToxR-activated genes (*tag*) exhibited some transcriptional activity even when grown under non-permissive conditions in the laboratory and, therefore, resolvase fusions to these *tag* genes were induced enough *in vitro* to result in resolution of the reporter in such newly constructed strains. This hypothesis was confirmed by the successful construction of unresolved *tag*::*tnpR* fusion strains (e.g. *acfB*::*tnpR* and *ctx*::*tnpR*) in a *toxR-null* genetic background (A. Camilli and J. J. Mekalanos, unpublished data).

As with most genetic screens or selections, only a subset of possible genes will be identifiable by any one method. In our case, we chose only those gene fusions that were initially transcriptionally silent or expressed at a low level during growth on a rich laboratory medium under aerobic conditions but were subsequently induced during infection. Our choice of a rich medium for the *in vitro* pre-screen was done to broaden the set of obtainable genes to include those that might be involved in general metabolism and nutrient scavenging in the host. Our protocol could easily be altered, with respect to both the pre-screening conditions as well as the infection model used, in order to identify other classes of genes. In addition, further modifications of our system can be envisaged that would broaden the set of identifiable genes to include those having higher basal levels of transcription. For example, the generation of gene fusions to a *tnpR* allele in which the ribosome-binding site has been mutated would reduce subsequent levels of resolvase expression. In addition, the incorporation of other selectable markers into the resolvase-excisible cassette (e.g. that allow positive selection of cassette-deleted progeny) should facilitate the recovery of rare resolved clones within the animal passaged pool. Because resolvase requires only a negatively supercoiled DNA substrate and Mg^{2+} as a cofactor (Reed, 1981), modified versions of our method should be broadly applicable for study of other bacterial pathogens, both Gram negative and Gram positive, and even, perhaps, unicellular eukaryotic pathogens.

The method described here has allowed us to identify 13 transcription units of *V. cholerae* that are specifically induced *in vivo* during infection. Our results demonstrate that only a subset of such transcripts will contribute measurably to virulence in animal infection models. However, we believe that a full understanding of host–parasite interactions must include the study of the complete set of pathogen genes expressed in response to host environments. The identification of pathogen gene products which are expressed during infection may facilitate the identification of new targets for antimicrobial drug development. The gene products expressed *in vivo* by pathogens may represent new, unrecognized targets for immune therapy or prophylaxis. Finally, the *in vivo*-induced promoters identified through these studies have applications in the expression of heterologous antigens in live, attenuated vaccines (J. R. Butterton, D. T. Beattie, C. L. Gardel, P. A. Carrol, T. Hyman, K. P. Killeen, J. J. Mekalanos and S. B. Calderwood, submitted).

Experimental procedures

Plasmid constructions

Plasmids are listed in Table 3. Plasmids pAC20 and pAC21, which are derivatives of pACYC184 (Chang and Cohen, 1978; Rose, 1988), were used to place the *res–tet–res* and *res1–tet–res1* sequences into the *V. cholerae* chromosome by allelic replacement of the endogenous *lacZ* gene. A 5.6 kb *AccI–EcoRV* fragment of the *V. cholerae* chromosome containing *lacZ* and surrounding sequences was inserted into the 2.55kb *Hin*cII–*Cla*I fragment of pACYC184 to generate pAC18. The 3.6kb *Pac*I–*Drd*I fragment containing *res–tet–res* was isolated from pRR51(Reed, 1981), treated with T4 DNA polymerase to blunt the DNA ends and had *Sac*I and *Kpn*I linkers (New England Biolabs) added sequentially. The resulting fragment was inserted into the unique *Kpn*I site of pAC18, which lay within the *lacZ* gene, to generate pAC20. The 3.6 kb *Kpn*I fragment containing *res1–tet–res1* was

isolated from pSKCAT2*tnpR* (Camilli *et al.,* 1994) and inserted into the unique *Kpn*I site of pAC18 to generate pAC21.

Plasmid pIVET5 is a derivative of pSKCAT2*tnpR* (Camilli *et al.,* 1994). Plasmid pSKCAT2*tnpR* was digested with *Sac*I, treated with T4 DNA polymerase to blunt the DNA ends and the 28bp *trpA* transcriptional terminator *trpAt* (Pharmacia) was inserted in the proper orientation to terminate RNA transcripts originating from the *tnpR* gene; this generated pSKCAT2*tnpRt*. The 6.2 kb *Bam*HI–*Nru*I fragment of pMC903 (Casadaban *et al.,* 1980) containing the *E. coli lacZYA′* sequences was treated with T4 DNA polymerase to blunt the DNA ends; *Kpn*I linkers (New England Biolabs) were added and the resulting fragment was inserted into the unique *Kpn*I site of pSKCAT2*tnpRt*. A recombinant plasmid, designated pIVET5, was isolated in which the transcriptional orientation of the inserted *lacZYA′* genes was the same as that of the *tnpR* gene.

Construction of bacterial strains and libraries

Bacterial strains are listed in Table 3. Allelic exchange of the chromosomal *lacZ* gene in the virulent streptomycin (Sm)-resistant parental *V. cholerae* strains O395 (classical biotype) and C6709-1 (El Tor biotype) was done using pAC21 and pAC20, respectively. Each plasmid was electroporated into *V. cholerae* and transformants in which the plasmids had integrated into the chromosomal *lacZ* gene were selected on LB agar (Sambrook *et al.,* 1989) containing 50 μg Sm, 2 μg Tc, and 3μg chloramphenicol (Cm). Several transformants were pooled and then passaged for approximately 20 generations in LB broth at 37°C. Beta-galactosidase-deficient allelic exchange mutant strains were screened for on LB agar containing 50 μg Sm, 1 μg Tc and 60 μg Xgal. Loss of plasmid-encoded chloramphenicol resistance (Cm^R) was confirmed for each of the allelic exchange mutant strains, which were designated AC-V66 (El Tor) and AC-V87 (classical).

The transcriptional gene fusion libraries in *V. cholerae* were constructed by first generating *Sau*3AI partial digests of total *V. cholerae* O395 and C6709-1 DNA. The partial digests were electrophoretically separated on a 1% low-melting-temperature agarose gel and the 1 to 3.5 kb range of fragments were isolated and then inserted into pIVET5 that had been cut with *Bgl*II and treated with calf intestinal alkaline phosphatase (New England Biolabs). Each pool of recombinant plasmids was electroporated into SM10λ*pir*. The resulting O395 and C6709-1-derived plasmid libraries were mobilized into AC-V87 and AC-V66, respectively, by biparental mating (Ditta *et al.,* 1980). Exconjugates in which the plasmids had integrated into the *V. cholerae* chromosome resulting in transcriptionally silent fusions to the *tnpR–lacZYA′* synthetic operon (Lac^-) were selected and then screened for on LB agar containing 100 μg Sm, 60 μg ampicillin (Ap) and 60 μg Xgal. Pools of 200 Lac^- colonies were collected by transferring an approximately equivalent portion of each colony to LB agar containing 2 μg Tc. Each agar plate was incubated at 37 °C for 2 h and the bacteria were collected in 1 ml of LB broth and stored at −70°C in 50% glycerol.

Each *in vivo*-induced gene fusion strain was reconstructed in a fresh genetic background. The integrated pIVET5 derivatives from each of the Tc^S strains recovered from the infant mice were able to excise out of the chromosome at the same low frequency at which they had originally integrated. These excised but non-replicating plasmids were recovered by

plasmid preparation from 100 ml of overnight LB broth cultures using Qiagen midi-prep columns (Qiagen) and using 1 μg tRNA as carrier for the isopropanol-mediated precipitation reaction. Each plasmid preparation was electroporated into SM10λ*pir* and transformants were selected for on LB agar containing 100 μg Ap. A single transformant from each electroporation was isolated and the plasmids were purified and their structures confirmed by restriction enzyme analysis (data not shown). Each plasmid was mobilized into either AC-V66 or AC-V87 by biparental mating as above, thus regenerating each gene fusion in the Tc^R *V. cholerae* strain backgrounds.

V. cholerae strains mutated in individual genes were constructed by inserting an internal coding fragment of DNA from each gene into pGP704 (Miller and Mekalanos, 1988). The pGP704 recombinant plasmids were electroporated into SM10λ*pir* and then subsequently mobilized into either O395 or C6709-1 by biparental mating. Exconjugates in which the plasmid had integrated into the chromosome were selected for on LB agar containing 100μg Sm and 60μg Ap. Correct integration into the chromosomal gene of interest was confirmed by Southern blot analysis (data not shown).

Initial screen for *V. cholerae* strains having *in vivo*-induced gene fusions

Each *V. cholerae* Lac^- gene fusion pool was grown to stationary phase at 30°C with aeration in LB broth containing 50 μg Sm, 30 μg Ap and 1 μg Tc. One 5-day-old infant CD-1 mouse (Charles River Breeding Laboratories) was intragastrically inoculated with 10^5 colony-forming units (cfu) per pool as previously described (Camilli *et al.,* 1994). The bacteria were recovered from the small intestines after 24 h as described (Camilli *et al.,* 1994) and approximately 1000 cfu were grown on LB agar containing 100 μg Sm. Tc^S strains were screened for by replica-plating the colonies onto LB agar containing 2 μg Tc and incubating the replica plates at 37°C for 8 h.

Quantification of resolution of *V. cholerae* gene fusion strains

Each of the reconstructed *V. cholerae* gene fusion strains was grown as above and used to inoculate 2 ml of 37°C LB broth with 4×10^4 cfu and to intragastrically inoculate infant mice as above. The LB broth cultures were grown 24 h at 37°C with aeration after which approximately 300cfu were grown on LB agar containing 100 μg Sm. Bacteria were recovered from the infant mouse small intestines after 24 h, as above, and approximately 300 cfu were grown on LB agar containing 100 μg Sm. The percentages of the bacterial populations recovered from the *in vitro* cultures and from the infant mice that were Tc^S were determined by replica-plating as above.

Competition assays

Each of the *V. cholerae* mutant strains to be tested and the fully virulent Lac^- wild-type strains AC-V167 and AC-V168 were grown to stationary phase at 30°C with aeration in LB broth containing 50 μg Sm and 30 μg Ap. A portion of each test strain culture was mixed with an equal volume of the appropriate wild-type strain culture and the resulting mixed culture was washed, diluted and approximately 10^5 cfu in total were used to intragastrically inoculate eight infant mice, as previously described (Camilli *et al.,* 1994). *In vitro* competitions were done by using each of the prepared inoculi above to inoculate 2 ml of

37°C LB broth with 4×10^4 cfu; after which the cultures were grown for 24 h at 37°C with aeration. The ratio of test strain to wild-type strain in each inoculum, as well as in the resulting bacterial populations recovered from the *in vitro* and from the *in vivo* competitions after 24 h, was determined by growing approximately 300cfu on LB agar containing 50 μg Sm, 30 μg Ap and 60 μg Xgal at 37°C and scoring the number of Lac^+ and Lac^- colonies after 24 h. A competitive index for each mutant strain tested was calculated as described in the legend to Table 2.

Sequencing of *tnpR* fusion junctions and flanking DNA

At least 300 bases of *V. cholerae* DNA flanking the 5′ end of *tnpR* was sequenced from each pIVET5 derivative recovered from the gene fusion strains, using primer 5′-GTTGATACCCGTGCGTAACC-3′, which hybridized to a sequence near the 5′ end of *tnpR* (Reed *et al.,* 1982). Sequencing was done using an ABI 373A automated sequencer according to the manufacturer's directions (Applied Biosystems Incorporated). In certain cases, *V. cholerae* DNA sequences 3′ of the fusion junction were obtained by inverse polymerase chain reaction (PCR) (Ochman *et al.,* 1988) from O395 or C6709-1 chromosomal DNA and sequenced as above using primers which hybridized to flanking *V. cholerae* sequences (data not shown).

Predicted amino acid sequences from all six reading frames of DNA sequence obtained above were subjected to similarity searches of the PIR-protein database (Release 43.0) using the FASTA program (Genetics Computer Group (GCG) version 7.3.1). Homologous polypeptide segments (see Figs 2, 3, 4 and 6) were compared using the BESTFIT program (GCG version 7.3.1) with a gap weight equal to 3 and a length weight equal to 0.1. *V. cholerae* DNA sequences which coded for the polypeptides segments shown in part A of Figs 2, 3, 4 and 6 were deposited into the GenBank database as *iviI* (database accession number U25709), *iviII* (U25710), *iviIII* (U25807), *ivIV* (U25711), *iviIV* (U25726), *orf4* (U25727), *orf5* (U25820), *orf3* (U25728), *iviVI* (U25729), *orf1* (U25730) and *iviXI* (U25731). The known bacterial proteins used for these comparisons were CysI (M23007), SucA (A30256), ArgA (A30372), NirT (S13937), Tar (A29053), *B. bronchiseptica* FlaA (A40594), *P. aeruginosa* FlaA (A37853), CheV (Z29584), CysA (A30301), HlyC (X16945), LipA (S25768), collagenase (S19658) and hypothetical 21.7kDa protein (P32892).

Beta-galactosidase activity assays

Beta-galactosidase assays were performed using a modification of the method of Miller (Miller, 1972): the method of Slauch and Silhavy (Slauch and Silhavy, 1991). The reaction was monitored by reading A_{420} on a microplate reader. Activities were calculated as $(U A_{600}^{-1}\ ml^{-1}$ cell suspension) $\times 10^3$, where U = μmol min^{-1} ONP formed. Assays were carried out on 37°C log-phase cultures of *V. cholerae* strain AC-V226 (*cysI*::*tnpR–lacZY*) grown in M9 minimal media (Sambrook *et al.,* 1989) supplemented with either 0.2% D-glucose, 0.2 mM $MgCl_2$, 1 mM glutathione and 1 mM O-acetyl-L-serine (inducing conditions) or 0.2% D-glucose, 0.2 mM MgCI2 and 0.2 mM L-cysteine (repressing conditions). Assays were carried out on 37°C log-phase cultures of *V. cholerae* strain AC-V228 (*argA*::*tnpR–lacZY*) grown in M9 minimal media supplemented with either 0.2% D-

glucose and 0.2 mM $MgCl_2$ (inducing conditions) or 0.2% D-glucose, 0.2 mM $MgCl_2$ and 0.2 mM L-arginine (repressing conditions).

Lipase activity assay

Secreted lipase activity was detected visually by formation of a clearing zone surrounding a heavily grown streak of *V. cholerae* after 48 h at 37 °C on LB agar supplemented with emulsified tributyrin. The media was prepared by adding 1 g Gum arabic (Sigma Chemical Co.) and 10 ml tributyrin (Sigma Chemical Co.) per litre of LB agar and emulsifying with a homogenizer prior to pouring the media.

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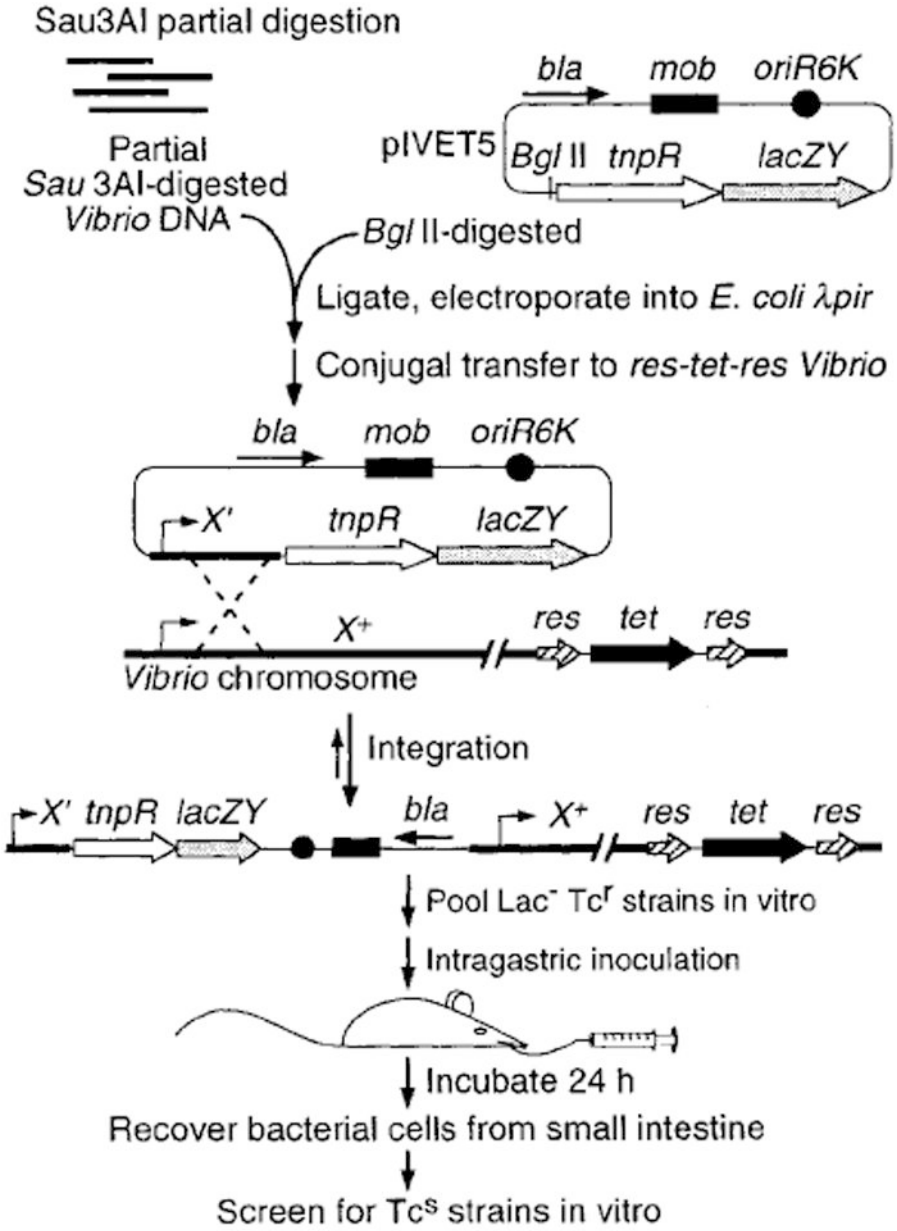

Fig. 1.

Schematic diagram of the screening protocol used to identify *V. cholerae* transcripts that are induced in the host. See the *Results* for details.

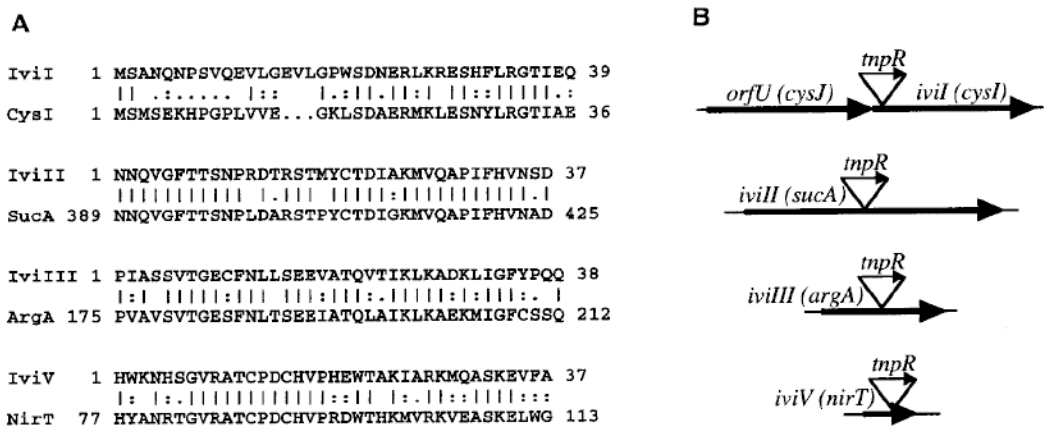

Fig. 2.

A. Similarities of deduced *V. cholerae* polypeptide partial sequences. Vertical lines indicate identical residues, highly conserved substitutions are represented by colons and conserved substitutions are represented by dots. Gaps were introduced into the sequences to maximize the similarities (see the *Experimental procedures*). For each *V. cholerae* polypeptide sequence shown, the C-terminal amino acid delineates the corresponding DNA site of fusion to *tnpR–lacZY*.

B. Schematic diagram (not to scale) of predicted *V. cholerae* genes containing fusions to *tnpR–lacZY* (designated by triangles). Transcriptional orientations are shown by the arrows.

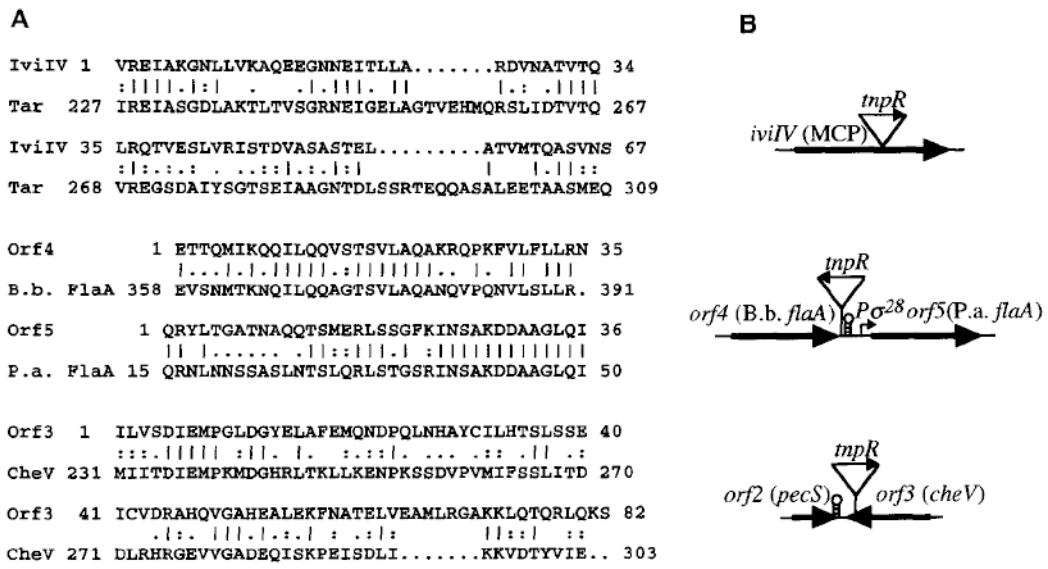

Fig. 3.

Similarities of deduced *V. cholerae* polypeptide partial sequences (A) and predicted genes (B). Details are as in the caption to Fig. 2, except that only for IvilV does the C-terminal amino acid delineate the corresponding DNA site of fusion to *tnpR–lacZY.* The DNA site of fusion to *tnpR–lacZY* in AC-V233 and AC-V234 occurred 51 bp 3′ of the *orf4* stop codon and within the codon for amino acid 45, respectively. Putative transcriptional terminators are designated by short stem–loop symbols. A putative σ^{28}-dependent promoter is shown immediately upstream of *orf5.* B. b. and P. a. refer to *Bordetella pertussis* and *Pseudomonas aeruginosa*, respectively.

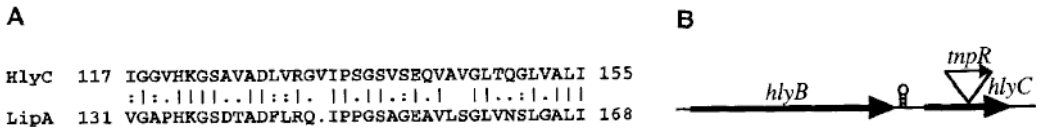

Fig. 4.

Similarities of deduced *V choterae* polypeptide partial sequences (A) and predicted genes (B). Details as in the legend to Fig. 2.

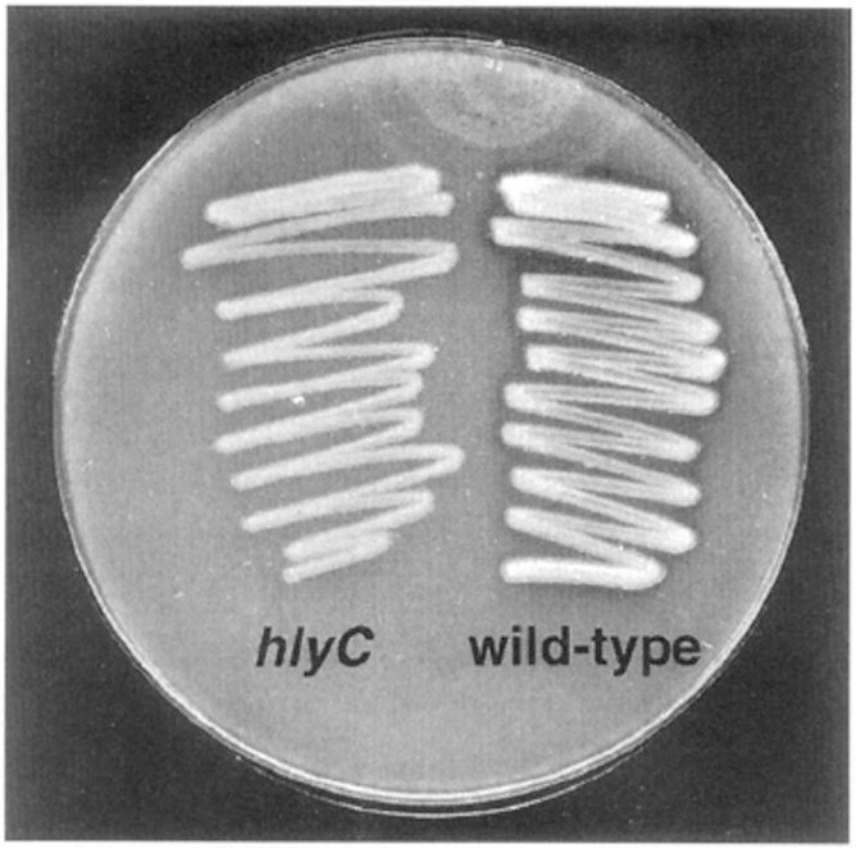

Fig. 5.

Secreted lipase activity from wild-type *V. cholerae* C6709-1 and the *hlyC* mutant AC-V195. The plate contained LB agar plus emulsified tributyrin and was incubated at 37°C for 48h.

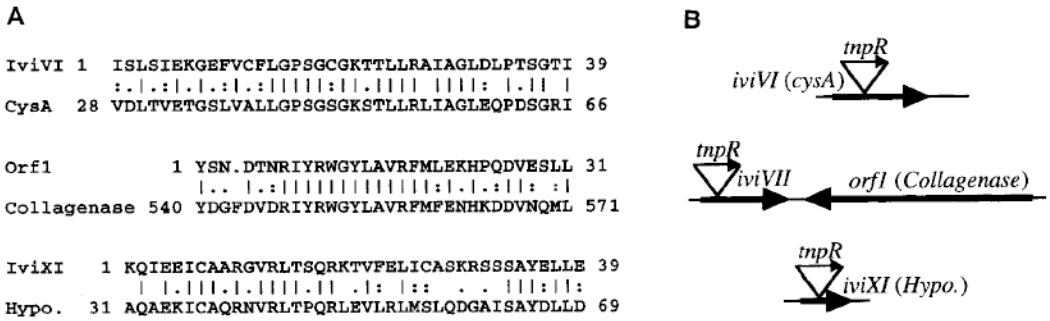

Fig. 6.

Similarities of deduced *V. cholerae* polypeptide partial sequences (A) and predicted genes (B). Details are as in the caption to Fig. 2 except that the N-terminal amino acid for IviVI delineates the corresponding DNA site of fusion to *tnpR–lacZY*.

Table 1

Resolution levels *in vitro* and *in vivo*.

	Tc^S (% cfu after growth)[a]			
Strain	***in vitro***	***in vivo***	***N*[b]**	***P*(<)[c]**
AC-V226	4	21	4	0.07
AC-V227	9	43	4	0.07
AC-V228	0	40	5	0.04
AC-V229	51	88	6	0.03
AC-V230	0	31	7	0.04
AC-V231	0	87	4	0.07
AC-V232	0	69	4	0.07
AC-V233	0	13	6	0.03
AC-V234	1	11	6	0.04
AC-V235	6	36	5	0.04
AC-V236	26	66	5	0.04
AC-V237	2	14	8	0.05
AC-V238	0	11	6	0.06

[a]Value given is the mean from 4–8 independent experiments.

[b]*N* equals the number of independent experiments, with each experiment consisting of two animals.

[c]*P* is the probability that each strain resolved to a greater extent (%Tc^S) in infant mice than when grown *in vitro,* using the Wilcoxon paired-sample test (Zar, 1974).

Table 2

Competition assays of *V. cholerae* mutant strains.

Test strain	Gene mutated	Competitive index[a]
AC-V172	*iviI*	1
AC-V176	*iviIII*	0.2[b]
AC-V179	*iviIV*	1
AC-V180	*iviV*	1
AC-V184	*iviVI*	1
AC-V186	*iviVII*	0.5[b]
AC-V187	*orf1*	0.9
AC-V188	*orf2*	1
AC-V189	*orf3*	2
AC-V191	*iviX*	0.9
AC-V193	*iviXI*	0.9
AC-V195	*iviXII*	0.7
AC-V196	*iviXIII*	0.3[b]

[a]Competitive indices from one representative experiment are shown for each strain. Each competitive index was calculated by dividing the ratio of test strain to wild-type bacteria from 6–8 infant mice outputs by that from the *in vitro* competition output, after correcting the ratios for deviations of the inoculum ratio from a value of 1. All 13 mutant strains had *in vitro* competitive ratios of approximately 1 and, therefore, did not have any general growth defects caused by the mutations (data not shown).

[b]$P<0.0078$ that the competitive indices were equal to or greater than a value of 1 using the sign test (Zar, 1974).

Table 3

Bacterial strains and plasmids.

Strain/Plasmid	Relevant genotype and phenotype	Source/Reference
Strain		
SM10λ*pir*	*thi recA thr leu tonA lacY supE* RP4-2-Tc::*Mu* λ::*pir*	Laboratory strain
DH5αλ*pir*	F⁻ Δ(*lacZYA–argF*)U169 *recA1 endA1 hsdR17 supE44 thi-1 gyrA96 relA1* λ::*pir*	Hanahan (1983); Kolter *et al.* (1978)
AC-V66	C6709-1 El Tor biotype, *lacZ::res–tet–res,* Sm^R	This work
AC-V87	O395 classical biotype, *lacZ::res1–tet–res1*, Sm^R	This work
AC-V226	AC-V66 *iviI*::pIVET5	This work
AC-V227	AC-V87 *iviII*::pIVET5	This work
AC-V228	AC-V87 *iviIII*::pIVET5	This work
AC-V229	AC-V66 *iviIV*::pIVET5	This work
AC-V230	AC-V87 *iviV*::pIVET5	This work
AC-V231	AC-V66 *iviVI*::pIVET5	This work
AC-V232	AC-V66 *iviVII*::pIVET5	This work
AC-V233	AC-V66 *iviVIII*::pIVET5	This work
AC-V234	AC-V66 *iviIX*::pIVET5	This work
AC-V235	AC-V66 *iviX*::pIVET5	This work
AC-V236	AC-V66 *iviXI*::pIVET5	This work
AC-V237	AC-V66 *iviXII*::pIVET5	This work
AC-V238	AC-V87 *iviXIII*::pIVET5	This work
AC-V167	O395 *lacZ*::pGP704	This work
AC-V168	C6709-1 *lacZ*::pGP704	This work
AC-V172	C6709-1 *iviI*::pGP704	This work
AC-V176	O395 *iviIII*::pGP704	This work
AC-V179	C6709-1 *iviIV*::pGP704	This work
AC-V180	O395 *iviV*::pGP704	This work
AC-V184	C6709-1 *iviVI*::pGP704	This work
AC-V186	C6709-1 *iviVII*::pGP704	This work
AC-V187	C6709-1 *orf1*::pGP704	This work
AC-V188	C6709-1 *orf2*::pGP704	This work
AC-V189	C6709-1 *orf3*::pGP704	This work
AC-V191	C6709-1 *iviX*::pGP704	This work
AC-V193	C6709-1 *iviXI*::pGP704	This work
AC-V195	C6709-1 *iviXII*::pGP704	This work
AC-V196	O395 *iviXIII*::pGP704	This work
Plasmid		
pGP704	*oriR6K mobRP4*, Ap^R	Miller and Mekalanos (1988)
pAC20	pACYC184::*lacZ*::*res–tet–res*	This work
pAC21	pACYC184::*lacZ*::*res1–tet–res1*	This work
pIVET5	pSKCAT2*tnpR*::*lacZYA' trpAt*	This work