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Broad-Host-Range Vectors for Delivery of Tn*phoA*: Use in Genetic Analysis of Secreted Virulence Determinants of *Vibrio cholerae*

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Gene fusions between the cholera toxin structural genes and *phoA*, which encodes bacterial alkaline phosphatase, were identified after Tn*phoA* mutagenesis of the cloned genes in *Escherichia coli* and were then mobilized into *Vibrio cholerae*. The activities of the hybrid proteins were detectable in *V. cholerae* and suggested that, like cholera toxin, they were secreted beyond the cytoplasm. To extend the utility of Tn*phoA* to identify additional genetic export signals in *V. cholerae* and other gram-negative bacteria, Tn*phoA* delivery vectors utilizing broad-host-range plasmids were developed. By using *V. cholerae* as a model system, insertion mutants carrying active *phoA* gene fusions were identified as colonies expressing alkaline phosphatase, which appeared blue on agar containing the indicator 5-bromo-4-chloro-3-indolyl phosphate. Since alkaline phosphatase is active only upon export from the cytoplasm, PhoA$^+$ colonies resulting from the mutagenesis procedure were enriched for insertions in genes that encode secreted proteins. Insertion mutations were identified in the gene encoding a major outer membrane protein, OmpV, and in *tcpA*, which encodes a pilus (fimbrial) subunit. Mutant strains harboring chromosomal insertions isolated in this manner can be used to assess the role of the corresponding inactivated gene products on survival of *V. cholerae* in vivo. The expression of the hybrid proteins as determined by measuring alkaline phosphatase activity also allowed the convenient study of virulence gene expression.

Vibrio cholerae is the gram-negative bacterial agent responsible for the acute diarrheal disease Asiatic cholera. The infectious process includes survival of the gastric acid barrier of the stomach, chemotaxis, and motility through the mucous layer lining the intestinal wall and subsequent colonization of the small bowel mucosa. These events are thought to be mediated by extracellular enzymes such as proteases and mucinases as well as surface structures such as flagella, pili, adhesins, and outer membrane proteins. Ultimately, elaboration of cholera toxin and possibly other secreted toxins (e.g., shigalike toxin and other cytotoxins) causes massive fluid loss and electrolyte imbalance in the host while possibly providing nutrients to and aiding in the subsequent dissemination of the bacterial agent. Thus, a constellation of secreted protein factors are utilized by *V. cholerae* during cholera pathogenesis. Similar protein virulence determinants are widely distributed among pathogenic microorganisms and likely have analogous functions in other bacterial diseases. To study the role of these virulence factors as well as perhaps previously unidentified ones, we sought to develop a general system to facilitate the isolation of defined mutations in virulence genes.

To accomplish these objectives, we developed a broad-host-range system for the efficient delivery and subsequent transposition into the chromosome of the recently described Tn*phoA* fusion vector (15). The *phoA* gene fusion approach is based on the fact that for the normally periplasmic protein bacterial alkaline phosphatase to be active it must be localized extracytoplasmically (5). Signal sequence mutations that block its export render the enzyme inactive (19). Thus, export is essential for high levels of alkaline phosphatase activity. It has been shown that export and activity can be concomitantly restored by fusing a restriction fragment containing a truncated *phoA* gene (lacking secretion signals) to portions of genes encoding signal sequences of heterologous proteins such as OmpF and LamB (10). Manoil and Beckwith have extended the utility of this approach by inserting a similar *phoA* restriction fragment near one end of Tn*5* to create a transposon, designated Tn*phoA*, that can randomly generate gene fusions to *phoA* upon insertion into a cloned target gene or the chromosome as outlined in Fig. 1. The hybrid proteins expressed by such gene fusions display PhoA activity only if the target gene encodes a membrane, periplasmic, outer membrane, or extracellular protein. Because such exported proteins represent the most frequent classes of proteins recognized to be involved in bacterial pathogenesis, the use of Tn*phoA* provides a strong enrichment for insertion mutations in virulence genes.

To introduce Tn*phoA* into *V. cholerae*, we placed the transposon onto mobilizable plasmids that allow for selection of transposition events after their introduction to the recipient. Due to the broad host range of both the delivery plasmids and Tn*5* transposition (2, 7, 23), the system is applicable to a wide range of gram-negative bacterial species. In addition, it can be used in appropriate *Escherichia coli* hosts to obtain fusions to virulence genes that naturally occur on plasmids or that have been previously cloned onto plasmids or bacteriophages (15).

MATERIALS AND METHODS

Bacterial strains, phages, and plasmids. The bacterial strains, phages, and plasmids used in this study and their relevant properties are listed in Table 1. Bacterial and phage growth, generalized transduction, DNA manipulations, and plasmid transformation were performed by standard methods (20, 29). Plasmids carrying Tn*phoA* were introduced into *V. cholerae* by conjugation as described below. Construc-

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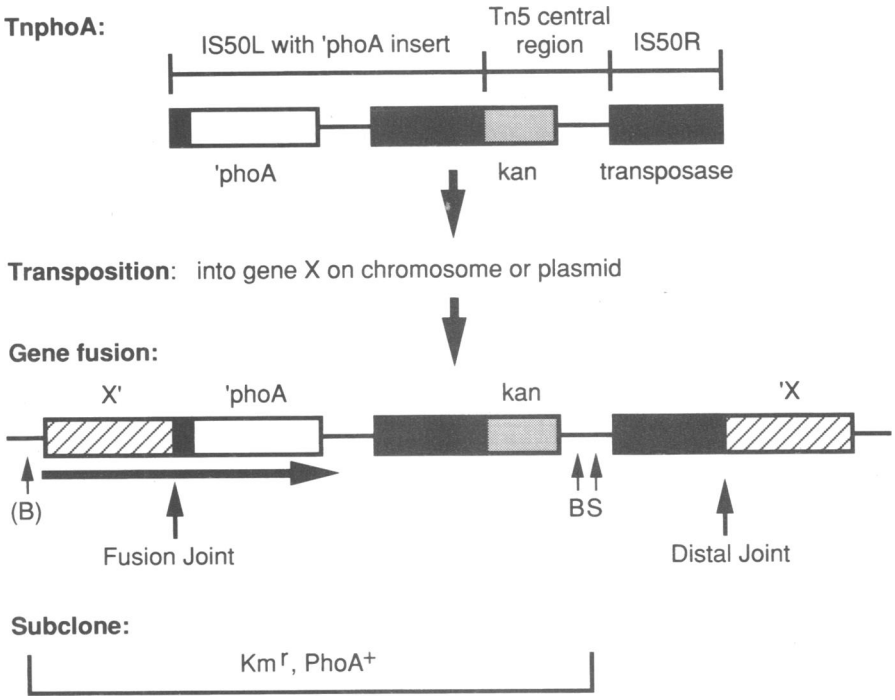

FIG. 1. Events leading to the formation and cloning of an active *phoA* gene fusion by using Tn*phoA*. The transposon is a derivative of Tn*5* with a region encoding *E. coli* alkaline phosphatase, minus the signal sequence and expression signals, inserted into the left IS*50* element (15). Active insertions into gene *X* interrupt the gene and result in production of a hybrid protein from the *X-phoA* fusion. One scheme for isolating the gene fusion is to utilize the *Bam*HI (B) or *Sal*I (S) sites that lie distal to the Kan^r gene and a hypothetical site (B) upstream of the gene to which *phoA* is fused. Restriction enzymes such as *Xba*I, *Stu*I, *Sac*I, and *Eco*RV, which do not cut within Tn*phoA*, are useful for isolating cloned fragments that carry the insertion plus DNA flanking both ends of the fusion joint.

tions on plasmid pBR322 were mobilized into *V. cholerae* by triparental matings employing the mobilizer plasmid pRK2013 (8).

Chemical reagents and enzymes. Growth media reagents were purchased from Difco Laboratories. Both *E. coli* and *V. cholerae* were stored in frozen 30% glycerol and grown in Luria broth (LB) or on LB agar (29). Antibiotics were purchased from Sigma Chemical Co. and used at the following concentrations; ampicillin, 100 μg/ml; gentamicin, 30 μg/ml; kanamycin, 30 or 300 μg/ml; streptomycin, 100 μg/ml; and tetracycline, 15 μg/ml. The chromogenic substrates 5-bromo-4-chloro-3-indolyl-phosphate (XP) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, used to detect alkaline phosphatase and β-galactosidase activity, respectively, on agar, were purchased from Bachem and incorporated into the agar at a final concentration of 100 μg/ml. Glucose supplements were at 0.2% final concentration. For in vitro DNA manipulations, restriction endonucleases and T4 DNA ligase were purchased from New England BioLabs, Inc., and used according to the manufacturer's suggestions. DNA sequencing was performed by the dideoxyoligonucleotide method (28) with Klenow fragment from International Biotechnologies Inc. or Amersham Corp. The oligonucleotide primer 5′ AATATCGCCCTGAGC 3′, which was used for sequencing the *phoA* fusion joints, was synthesized by New England BioLabs.

Isolation of active *phoA* fusions on plasmids. The previously described F42 *lacI3 zzf-2*::Tn*phoA* (15) was used as a source of Tn*phoA* to create fusions to *V. cholerae* toxin genes (*ctxAB*) cloned into multicopy plasmids in *E. coli*. Briefly, the *ctxAB*$^+$ plasmids were first transformed into the *phoA* mutant CC118 carrying the F factor. Transformants were suspended in LB, and several dilutions were plated on agar containing ampicillin to select for maintenance of the plasmid and a high concentration of kanamycin (300 μg/ml) to enrich for cells carrying insertions of Tn*phoA* into the multicopy plasmid. In addition, XP was included in the agar to score for colonies expressing active hybrid proteins. Blue colonies were purified, and plasmid DNA was prepared and used to transform CC118 to Ap^r and Km^r (30 μg/ml) on agar containing XP to again score for colonies expressing hybrid proteins with alkaline phosphatase activity. Loss of target gene function and restriction analysis were used to localize the position of the Tn*phoA* insertion into these plasmids.

Construction of vectors for the delivery of Tn*phoA* to *V. cholerae*. Plasmid vectors designed to deliver Tn*phoA* into a broad range of gram-negative bacteria were isolated as described above to enrich for the presence of Tn*phoA* on multicopy plasmids. However, in this case, the resulting plasmid harboring Tn*phoA* was isolated from a white colony which was therefore not expressing an active fusion. Plasmid pRT291 was isolated by using pRK290 (8) as the starting plasmid and selecting for cotransformation of Tc^r and Km^r after enrichment. Plasmid pRT733, a derivative of the suicide vector plasmid pJM703.1 (22), was selected as Ap^r Km^r.

Isolating active *phoA* fusions in *V. cholerae* by using the pRT291 and pRT733 plasmid vectors. Figure 2 depicts the scheme used for isolating active *phoA* fusions in *V. cholerae*. The first step was to conjugate the particular vector into a streptomycin-resistant derivative of *V. cholerae* O395 or 569B of the classical biotype or E7946 of the El Tor biotype. For pRT291, fresh colonies of donor strain SM10 (30) carrying the vector were mated with recipients on LB agar by cross streaking and then incubating at 37°C for 6 to 24 h. The mating mix was then streaked or spread after dilution onto agar containing either streptomycin plus tetracycline or

TABLE 1. Bacterial strains, plasmids, and bacteriophage

Strain, plasmid, or phage	Genotype or characteristics	Reference or source
E. coli		
CC118	*araD139* Δ(*ara leu*)*7697* Δ*lacX74* Δ*phoA20 galE galK thi rpsE rpoB argE*(Am) *recA1*	15
JF626	Δ(*lac pro*) *thi rpsL supE endA sbcB15 hsdR4* (F′ *traD36 proAB lacI*^{q} *lacZM15*)	J. Felton
MC1061	Δ(*ara leu*)*7697* Δ*lacX74 galE galK hsdR rpsL*	6
MM294	*endA hsdR pro supF*	27
SM10	*thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu::	30
V. cholerae		
569B	Classical, Inaba Str^r derivative	Laboratory collection
E7946	El Tor, Ogawa Str^r derivative	Laboratory collection
O395	Classical, Ogawa Str^r derivative	Laboratory collection
569BW1	569B Str^r *phoX*	This study
569B2.2	569B Φ(*ompV-phoA*)*2.2*(Hyb)	This study
RT110.21	O395 Φ(*tcpA-phoA*)*21*(Hyb)	This study
Plasmids		
F42Tn*phoA*	F42 *lacI3 zzf-2*::Tn*phoA*	15
pBR322	*oriColE1* Amp^r Tet^r	4
pJM703.1	*oriR6K mobRP4* Amp^r	22
pPH1JI	*traIncP1* Spc^r Gen^r Str^r	3
pRK290	IncP1 Tet^r	8
pRK2013	*traIncP1 ori* ColE1 Kan^r	8
pRT41	pBR322 *ctxAB*	This study
pRT41-16	pRT41 Φ(*ctxA-phoA*)*16*(Hyb)	This study
pRT46	pBR322 *ctxB*	This study
pRT46-3.2	pRT46 Φ(*ctxB-phoA*)*3.2*(Hyb)	This study
Bacteriophage		
λ *pir*	*pirR6K*	12
M13mp10		18

streptomycin plus tetracycline and kanamycin, since all transconjugants should acquire both Tc^r and Km^r due to the plasmid and Tn*phoA*, respectively. To select for transpositions of Tn*phoA*, independent colonies were subsequently mated with MM294 carrying the gentamicin-resistant plasmid pPH1JI, which is incompatible with pRK290 (3). The mating mix was plated on agar containing gentamicin, kanamycin, and streptomycin, thereby simultaneously selecting for pPH1JI and retention of Tn*phoA*. All of the resulting colonies were Tc^s and carried Tn*phoA* transposed from the pRT291 vector. The indicator XP was included in the agar to score for those transpositions that resulted in active *phoA* gene fusions. It often took several days for visualization of some of the weakly expressed fusions.

The vector pRT733 was introduced into the same strains of *V. cholerae* in the same manner as above except that the donor strain was SM10 λ *pir*. Plasmid pRT733 is a suicide vector, since it cannot replicate without the π protein encoded by the λ *pir* transducing phage (12, 22). Therefore, selection for Tn*phoA* upon transfer of pRT733 into *V. cholerae* directly resulted in the isolation of transpositions of Tn*phoA* into the chromosome. Transconjugants were selected in a single step on agar containing kanamycin, streptomycin, and XP.

Cloning and DNA sequencing *phoA* fusion joints. The appropriate gene fusions were first cloned into pBR322 (4). Two micrograms of total DNA isolated from strain 569B2.2 (*ompV-phoA*) or from strain RT110.21 (*tcpA-phoA*) was digested with *Bam*HI, ligated with 0.2 μg of similarly digested pBR322, and transformed into strain MC1061 with selection for Ap^r and Km^r on agar containing XP to confirm that clones arising from this selection were expressing active gene fusion products. To sequence the portion of *ompV* proximal to the fusion joint, the pBR322-derived clone was digested with *Bam*HI, which cleaves upstream of *ompV*, and *Pvu*II, which cleaves within *phoA*, and ligated with M13mp10 DNA previously digested with *Bam*HI and *Hin*cII. Single-stranded DNA isolated from phage that yielded white plaques containing the correct insert as deduced from restriction analysis of the replicative form was sequenced by the dideoxyoligonucleotide method (28) and primed with an oligonucleotide that hybridizes to *phoA* sequences just downstream of the fusion joint, similar to strategies utilized by others to obtain sequence of DNA adjacent to transposon insertions (14). The sequence of *tcpA* was determined in a similar manner, except that the cloned DNA in pBR322 was digested with *Bam*HI, which cleaves upstream of *tcpA*, and *Pst*I, which cleaves downstream of *phoA*, and cloned into similarly digested M13mp10. In this case plaques that were blue in the presence of XP, indicating the expression of an active *phoA* fusion, were selected for sequence analysis.

Mutagenesis. To obtain mutant strains that did not express endogenous phosphatase activity, *V. cholerae* 569B was mutagenized with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine as described previously (20, 29), and survivors were plated on agar containing XP. To discern phosphatase mutants from those defective in XP uptake, several of the isolates were screened for blue color on XP after mating in a plasmid carrying a *phoA* fusion known to express in *V. cholerae*.

Quantitation of alkaline phosphatase activity. To measure the amount of alkaline phosphatase produced by the fusion-carrying strains, cultures were grown in LB for 8 to 16 h. Cells were harvested and suspended in 1 M Tris hydrochloride (pH 8), a sample was diluted 1/20 and permeabilized with sodium dodecyl sulfate and chloroform, and the rate of hydrolysis of *p*-nitrophenyl phosphate was measured as described previously (19). Culture supernatants were as-

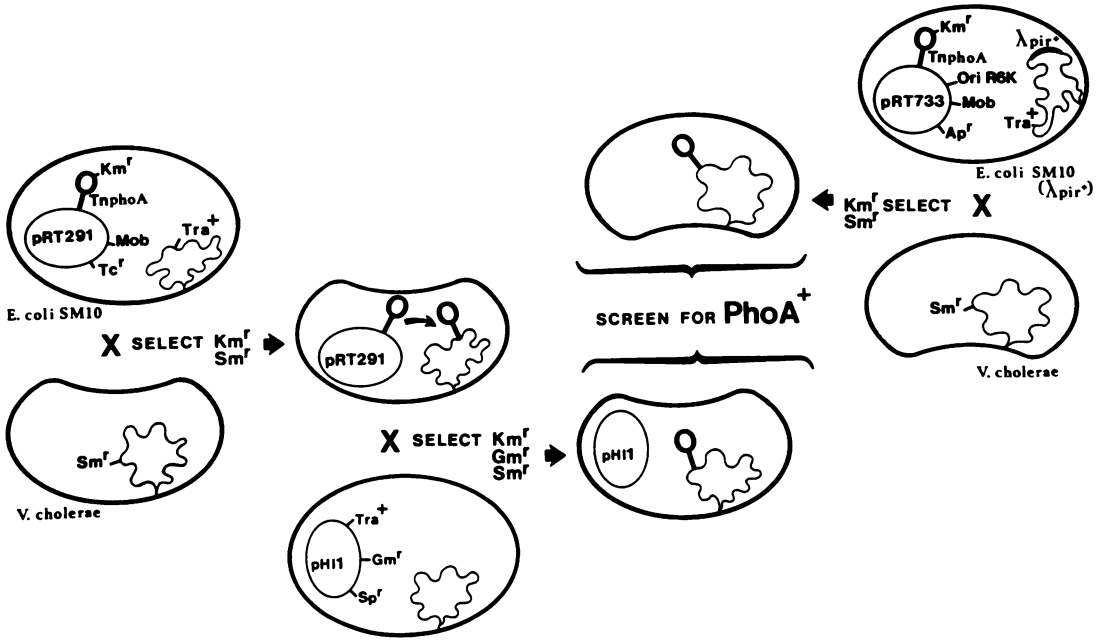

FIG. 2. Two methods for delivery and selection of transposition of Tn*phoA* from *E. coli* to other gram-negative bacteria. On the left, pRT291 which carries Tn*phoA* is mobilized into an Sm^r *V. cholerae* recipient strain. Exconjugants are then utilized as the recipients for a second plasmid, pHI1, of the same incompatibility group as pRT291 and encoding Gm^r. By maintaining selection for the presence of Tn*phoA* (Km^r), colonies are obtained in which Tn*phoA* has transposed and the vector plasmid has been lost. Colonies resulting from a fusion that expresses active alkaline phosphatase appear blue when XP is incorporated into the agar. The second method, shown on the right, utilizes the suicide vector pRT733, which allows for selection of transpositions in a single step. The vector is mobilized into a *V. cholerae* recipient, and selection for the acquisition of Tn*phoA* (Km^r) directly yields transpositions since pRT733 cannot replicate without the functions provided in *trans* by λ *pir*.

sayed in the same manner after a 1/20 dilution of spent medium into 1 M Tris hydrochloride (pH 8).

Quantitation of cholera toxin B subunit production. To screen for fusions in the cloned toxin genes, toxin production was determined for cultures grown in LB overnight. The toxin concentration was determined by the addition of the broth or a freeze-thaw cell lysate to a GM1 ganglioside-specific enzyme-linked immunosorbent assay as described previously (11).

RESULTS

Fusions to the toxin structural genes, *ctxA* and *ctxB*. To confirm that the enzymatic and localization properties that PhoA hybrid proteins display in *E. coli* are maintained in *V. cholerae*, we first constructed Tn*phoA* fusions to the cloned cholera toxin structural genes (17, 25). The *ctxA* and *ctxB* genes each encode separate N-terminal signal sequences and thus were expected to yield PhoA$^+$ Tn*phoA* fusion proteins. Two plasmids containing subclones of the toxin genes were constructed to optimize target size with respect to the rest of the plasmid and to allow for subsequent mobilization into *V. cholerae*. One plasmid, pRT41, carries both the *ctxA* and *ctxB* genes, including the wild-type promoter inserted between the *Bam*HI and *Eco*R1 sites in pBR322 in the counterclockwise orientation. A second plasmid, pRT46, carries only *ctxB*, which is inserted in the *Bam*HI site of pBR322 and utilizes the *tet* promoter for expression. Its product, CtxB, is efficiently secreted extracellularly by *V. cholerae* but remains in the periplasm of *E. coli*, similar to holotoxin (25). Active *phoA* gene fusions were identified in both plasmids by using enrichment for transposition of Tn*phoA* for F Tn*phoA* into multicopy plasmids (15).

Five independent fusions were isolated in *ctxA* on pRT41; all mapped to different positions as determined by restriction mapping and DNA sequencing of the fusion joints for the shortest and longest. As expected, all of the fusion joints occurred distal to the signal sequence processing site. The shortest hybrid protein contains only the first 11 residues of native toxin A subunit and is designated Φ(*ctxA-phoA*)*16*(Hyb), corresponding to plasmid pRT41-16. This fusion was chosen for further study since it contains essential export information and does not express portions of CtxA required for toxin activity. The largest fusion occurred in the codon corresponding to residue 134, which is a little more than half way through the protein. To isolate fusions in *ctxB*, plasmid pRT46 was utilized. Nine independent fusions that mapped throughout *ctxB* were identified. The shortest occurred at residue 5 of the native protein, and the longest occurred 8 residues from the end. The longest fusion, Φ(*ctxB-phoA*)*3.2*(Hyb) on plasmid pRT46-3.2, was chosen for further study since it contained the most material that might be required for extracellular export by wild-type *V. cholerae*.

Plasmids pRT41-16 and pRT46-3.2 were mobilized into wild-type *V. cholerae* 569B, and the expression of the hybrid proteins was measured by assaying cell-associated alkaline phosphatase activity after overnight growth in LB. In addition, the culture supernatant was assayed for activity to determine whether the fusion proteins might be extracellularly localized. Both fusions exhibited high levels of alkaline phosphatase in *V. cholerae* (Table 2). The *ctxA-phoA* fusion is under control of its own promoter and requires the *V. cholerae* ToxR protein for maximal expression (21). Consistent with this, the alkaline phosphatase activity of the fusion on pRT41-16 increased upon mobilization from *E. coli* to *V. cholerae*, whereas that of the *ctxB-phoA* fusion, which is under transcriptional control of the *tet* promoter on pBR322, showed less than twofold increased activity in *V. cholerae*.

TABLE 2. Alkaline phosphatase activities exhibited by *phoA* gene fusions when present in *E. coli* and *V. cholerae*

Strain	Fusion	Alkaline phosphatase activity (U)	
		Cell associated	Culture supernatant
E. coli			
CC118		3	8
CC118(pRT41-16)	Φ(*ctxA-phoA*)*16*(Hyb)	93	3
CC118(pRT46-3.2)	Φ(*ctxB-phoA*)*3.2*(Hyb)	379	6
CC118(pRT2.2)	Φ(*ompV-phoA*)*2.2*(Hyb)	467	17
V. cholerae			
569B		5	7
569BW1		6	5
O395		4	7
569B(pRT41-16)	Φ(*ctxA-phoA*)*16*(Hyb)	626	10
569B(pRT46-3.2)	Φ(*ctxB-phoB*)*3.2*(Hyb)	727	18
569B2.2	Φ(*ompV-phoA*)*2.2*(Hyb)	1,103	742
569B(pRT2.2)	Φ(*ompV-phoA*)*2.2*(Hyb)	1,378	1,808
RT110.21	Φ(*tcpA-phoA*)*21*(Hyb)	905	74
RT110.21 *toxR55*	Φ(*tcpA-phoA*)*21*(Hyb)	144	10

It is also evident from these assays that the phosphatase activity of both fusion proteins remained cell associated in both bacterial species (i.e., little activity was detected in the supernatant fluids of *V. cholerae* or *E. coli*). Since CtxB is normally extracellularly secreted by *V. cholerae*, this result suggests either that a domain of CtxB required for extracellular localization is deleted or altered in the CtxB-PhoA fusion protein or that the PhoA moiety cannot readily passage through the outer membrane. The cellular location of the CtxA-PhoA and CtxB-PhoA fusion proteins was not determined, but because of their high enzymatic activity it is likely that these hybrid proteins have been transported across the cytoplasmic membrane at a minimum and therefore reside in the periplasm or outer membrane of both *E. coli* and *V. cholerae*.

Detection on agar plates of *phoA* gene fusion activity in *V. cholerae*. Extending the utility of Tn*phoA* to *V. cholerae* relies on the detection of active fusions through the incorporation of the chromogenic indicator XP into the plating medium. When *V. cholerae* was grown on L agar containing XP, the colonies exhibited a pale blue color, which could potentially hinder the detection of *phoA* fusions of low activity. Interestingly, the *V. cholerae*-encoded phosphatase responsible for cleaving XP did not appear to cleave *p*-nitrophenol phosphate in the assay used to quantitate alkaline phosphatase activity of bacteria after growth in broth (Table 2). Two methods were used to eliminate the background of XP cleavage in agar. The first method was to isolate strains that lacked the phosphatase activity. Strain 569B was mutagenized with nitrosoguanidine, and white colonies were identified after growth on L agar with XP. About 80% of these colonies were defective in the uptake of XP, since they remained white when pRT46-3.2 was mobilized into them. However, several phosphatase-negative isolates were identified that exhibited intensities of blue color and amounts of alkaline phosphatase activity (data not shown) similar to those of the parent strain when pRT41-16 or pRT46-3.2 was introduced. One of these, 569BW1, represents an altered *V. cholerae* strain suitable for Tn*phoA* mutagenesis.

An inherent problem with the above approach is the possibility of additional undetected mutations due to the mutagenesis procedure. Therefore, we also utilized a second method to lower the background color. During our search for mutants lacking activity on XP, we found that when *V. cholerae* is plated in the presence of a fermentable carbon source, i.e., glucose, maltose, or sucrose, the enzymatic activity that cleaves XP is decreased to a nondetectable level. The lack of activity was determined not to be due to catabolite repression, since many sugars caused the effect and adding cyclic AMP or dibutyryl cyclic AMP to the agar did not reverse it (data not shown). However, buffering the agar with 10 mM Tris hydrochloride (pH 7.5) restored the background phosphatase activity in the presence of the fermentable carbon sources, suggesting that the lack of activity is due to the decrease in pH when the colony metabolizes the sugar. Indeed, during the isolation of chromosomal fusions an insertion mutant was isolated that exhibits a blue phenotype and also enables all the other colonies in its vicinity to turn blue. This mutant does not harbor an active alkaline phosphatase fusion when assayed with *p*-nitrophenyl phosphate, but it seems to turn blue by virtue of having acquired the ability to alter the pH of the agar surrounding it toward basic as measured by placing strips of pH indicator paper on the agar plate.

Since the endogenous phosphatase activity is not detectable as measured by its ability to cleave *p*-nitrophenylphosphate under the conditions used to assay the *E. coli* alkaline phosphatase, it may be another type of phosphatase. We have designated the gene defined by the mutation in strain 569BW1 as *phoX*. Neither the nature of the enzyme nor the mutation has been characterized further. Growth on glucose was used for the subsequent isolation of Tn*phoA* fusions in *V. cholerae* since it did not require any prior mutagenesis of the strains. However, for other bacterial species, mutations identified in the above manner that inactivate endogeneous phosphatases may be a prerequisite for the detection of active fusions.

Construction of vectors for delivery of Tn*phoA* into *V. cholerae*. Since Tn*5* and its derivatives are able to transpose efficiently and with little sequence specificity in many gram-negative bacteria (2), we chose to create delivery systems consisting of broad-host-range plasmids harboring Tn*phoA*. The first used pRK290, an IncP plasmid derived from RK2 (8), as a vector plasmid that can subsequently be displaced with a second IncP plasmid while maintaining selection for Tn*phoA*, thus enriching for transposition. The second parent plasmid, pJM703.1, is a suicide vector derivative of pBR322 originally developed to facilitate marker exchange experiments in *V. cholerae* (22). In this plasmid the ColE1 origin of replication has been replaced by the *cis* required components of the R6K *ori*, and thus it can only replicate when the protein is provided in *trans* such as in an *E. coli* λ *pir* lysogen (12). In addition, pJM703.1 carries the mobilization site of RP4 (30). As such, both pRK290 and pJM703.1 are able to be mobilized by helper plasmid pRK2013 (8) or when present in strain SM10, which provides P-group transfer functions expressed from RP4-2-Tc::Mu stably integrated into the chromosome (30).

To construct derivatives of the plasmids carrying Tn*phoA*, the parent plasmids were transformed into strain CC118 F Tn*phoA* or the corresponding λ *pir* lysogen when using pJM703.1. After enrichment for transposition of Tn*phoA* into the multicopy plasmids (15), plasmid DNA was prepared and used to transform strain CC118, or CC118 λ *pir*, with coselection for both the appropriate parent plasmid and Tn*phoA*. Transformants were selected on agar containing XP to visualize colonies arising from bacteria harboring plasmids with Tn*phoA* inserted in a manner that did not lead

to the expression of an active hybrid protein (white colonies). Representative clones were purified and tested for 100% linkage between the parent plasmid and TnphoA by selecting for the resistance conferred by the parent and scoring for Km^r. A resulting vector of each type was isolated and designated pRT291 for the pRK290 derivative and pRT733 for the pJM703.1 derivative. Their general structure is shown in Fig. 2.

Transposition of TnphoA in *V. cholerae*. Figure 2 summarizes the procedures used to obtain fusions with the two types of TnphoA vector plasmids in the *V. cholerae* strains listed in Table 1. We first chose pRT291. Strain SM10(pRT291) yielded a high mating efficiency, with about 20% of the recipient cells receiving the plasmid when selected on agar containing tetracycline, kanamycin, and streptomycin. The second step in using pRT291 was to select for cells where TnphoA had transposed onto the chromosome and to screen for those containing active fusions. This was accomplished by introducing a plasmid of the same incompatibility group as pRT291 while retaining selection for TnphoA, similar to the method previously used by Ruvkun and Ausubel to recombine Tn*5* insertions in cloned genes back onto the *Rhizobium* chromosome (27). Strain MM294, carrying the self-transmissable plasmid pPH1JI (3), also designated pHI1 as in Fig. 1, was mated with the pRT291 containing *V. cholerae* strains. Transconjugants were selected on L agar containing gentamicin, kanamycin, streptomycin, XP, and 0.2% glucose. Transpositions occurred on an average of 1 in every 10^4 cells harboring pRT291 for the classical strains 569B and O395 and 1 in 10^5 for the El Tor strain E7946. Twenty independent pools of exconjugates yielded active fusions in 0.5 to 2% of the cells plated, detected as blue colonies after 1 to 3 days of incubation.

The pPH1JI plasmid is not maintained efficiently during growth at 42°C. After one round of overnight growth at 42°C, about 20% of the colonies were Gm^s and had lost the plasmid, as determined by agarose gel analysis of DNA isolated from several such derivatives. Such colonies were used for further studies, although the presence of pPH1JI has not seemed to affect any phenotypes that we have investigated.

The second method used for isolating *phoA* gene fusions in *V. cholerae* was through the use of the suicide vector pRT733. Transconjugants and transposition events were selected in a single step with about 1 in 10^6 recipient cells becoming Km^r and again 0.5 to 2% of those resulting in blue colonies. Although this vector system can yield transpositions in a single step, it often yielded Ap^r colonies. Such strains, presumed to arise from cointegrate formation between the vector and the chromosome since plasmids conferring Ap^r and Km^r cannot be isolated from them, occurred in about 20% of the *V. cholerae* colonies harboring active fusions. This frequency appears to be species dependent (R. K. Taylor, unpublished results).

Identification of an active *phoA* fusion to *ompV*, a gene encoding a major outer membrane protein of *V. cholerae*. The major outer membrane proteins are among the most abundant exported proteins present in a bacterial cell and thus represent targets for easily detectable hybrid PhoA fusion proteins. Therefore, crude outer membrane-enriched fractions were prepared (24) from several of the most highly expressing fusion isolates after TnphoA mutagenesis of strain 569B using pRT291. These fractions were boiled in loading buffer and run on a sodium dodecyl sulfate-polyacrylamide gel to visualize any alterations in the outer membrane protein profile. One of these strains is missing the 25-kilodalton major outer membrane protein previously designated as OmpV (31) (Fig. 3, lane B). This strain, designated 569B2.2, harbors the most active fusion thus far isolated, producing nearly 2,000 U of alkaline phosphatase from an overnight culture. Approximately 60% of this activity was cell-associated, with the other 40% free in the culture supernatant after overnight growth (Table 2). To determine whether strain 569B2.2 did indeed carry an insertion of TnphoA in the *ompV* structural gene, and not in another gene that might alter its expression, we utilized several properties of *phoA* fusions to clone a chromosomal DNA restriction fragment containing the fusion joint. By utilizing the cloning strategy shown in Fig. 1, *Bam*HI-digested DNA from strain 569B2.2 was ligated with similarly digested pBR322 and used to transform *E. coli* MC1061 with selection for Ap^r, Km^r, and blue colonies in the presence of XP. Restriction analysis of the DNA fragment adjacent to *phoA* matched that previously reported for *ompV* (31), indicating that the fusion was likely to be within this gene.

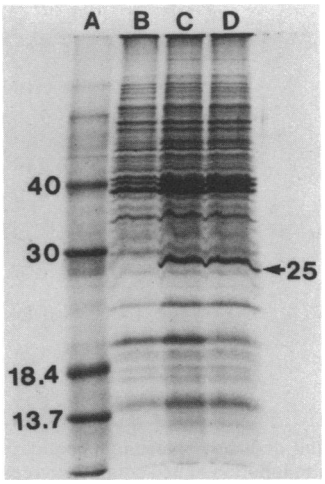

FIG. 3. Protein profile of a crude membrane enriched fraction from wild-type and *phoA* fusion-containing strains of 569B subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. (A) Molecular weight standards, (B) 569B Φ(*ompV-phoA*)*2.2*(Hyb), (C) 569B, (D) a random PhoA$^+$ fusion strain.

To further characterize *ompV* and the determinants leading to the localization of the hybrid protein, the DNA sequence of the *ompV-phoA* fusion joint was determined after subcloning of the *ompV-phoA* fusion junction into M13mp10 (18). The sequence of the coding portion of *ompV* and the derived amino acid sequence of the protein are shown in Fig. 4. This sequence is in agreement with that recently published for *ompV* (26).

Properties of OmpV that are typical of secreted proteins. The deduced primary structure of the amino-terminal portion of OmpV revealed a stretch of uncharged amino acids that yield a hydrophobic region (Fig. 5). Such a structure is typical of signal sequences found in many exported proteins (33). In fact, a predicted signal peptidase cleavage site is present in OmpV just past the hydrophobic region and indicated by the arrow in Fig. 4. In addition to these features derived from the amino acid sequence analysis, the secretory properties of the fusion protein suggest that other domains critical for localization are located before the fusion joint. As noted above, 40 to 60% of the alkaline phosphatase activity exhibited by the fusion is present in the culture supernatant. However, in *E. coli* strains carrying the same plasmid with the *ompV-phoA* fusion, the activity is 95% cell associated (Table 2). This property was retained in cells that

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                                        27                                  54
ATG AAA AAG ATC GCA CTA TTT ATC ACA GCA TCA CTT ATC GCA GGC AAC GCT CTA
MET Lys Lys Ile Ala Leu Phe Ile Thr Ala Ser Leu Ile Ala Gly Asn Ala Leu
                                        81                                  108
GCT GCC CAA ACT TAC ATT CGT AAC GGC AAT ATC TAT ACC CAC GAA GGT CAA TGG
Ala Ala Gln Thr Tyr Ile Arg Asn Gly Asn Ile Tyr Thr His Glu Gly Gln Trp
  ▲                                     135                                 162
GCT GCG GAA GTG GGT GCT TTT GGC AGT ACT GAC CTA CTC AAG GAT CAA GAC AAA
Ala Ala Glu Val Gly Ala Phe Gly Ser Thr Asp Leu Leu Lys Asp Gln Asp Lys
                                        189
TCT TAC GGT GCT TTA CTG AAC TTT GGT TCT
Ser Tyr Gly Ala Leu Leu Asn Phe Gly Ser
```

FIG. 4. DNA and deduced N-terminal sequence of *ompV* determined from a clone of the (*ompV-phoA*)*2.2*(Hyb) fusion. The last Ser is created by the fusion junction. The sequence agrees completely with that of Pohlner et al. (26). The fusion occurred downstream of the signal sequence processing site marked by the vertical arrow, indicating the ability of Tn*phoA* to identify *V. cholerae* chromosomal genes that encode secreted proteins.

had a normal outer membrane protein content (retained OmpV) and was not simply due to general leakage out of the membrane that was defective due to the lack of OmpV, since the cloned *ompV-phoA* fusion on a plasmid in wild-type 569B yielded 60% of the alkaline phosphatase activity to the culture supernatant (Table 2). This suggests that, besides containing a signal sequence, the N-terminal portion of OmpV present in the fusion protein may encompass a domain that is critical for localizing the fusion protein to the outer membrane of *V. cholerae*, where it is subsequently released into the culture. This domain does not appear to function in *E. coli*.

Isolation of Tn*phoA* insertions in a gene that encodes a major pilin subunit. An immediate test of the application of Tn*phoA* in the identification of previously uncharacterized genes encoding putative virulence determinants was to isolate an insertion in the *tcpA* gene, which encodes the major subunit of the toxin-coregulated pilus (TCP) elaborated by *V. cholerae* (32). Specifically, we sought to obtain a pair of strains, with one being the wild-type strain *V. cholerae* O395 that is virulent in both humans and animal models and the second differing only with respect to a single mutation knocking out expression of a functional *tcpA* product. Our screen for such mutants took advantage of the fact that *V. cholerae* bacteria that optimally express the TCP pilus autoagglutinate in broth culture and of the specificity of active fusions obtained with Tn*phoA* to yield insertions in genes encoding secreted products. By screening a total of only 40 PhoA$^+$ mutants from 12 independent pools, seven strains were identified that no longer autoagglutinated. Total protein profiles of these strains displayed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels showed that four independent mutants had specifically lost only the 20.5-kilodalton pilin protein (shown for one mutant, RT110.21, in Fig. 6). These four fusions were then cloned by using the strategy shown at the bottom of Fig. 1, and the fusion junction DNA was sequenced by utilizing the dideoxynucleotide method and a primer to the *phoA* portion of the fusion vector (C. Shaw and R. Taylor, unpublished results). In the case of all four fusions, the adjacent sequence upstream of the fusion joint matched that of the amino-terminal protein sequence previously determined for the TcpA pilin (32), demonstrating that the insertions were indeed within the structural gene.

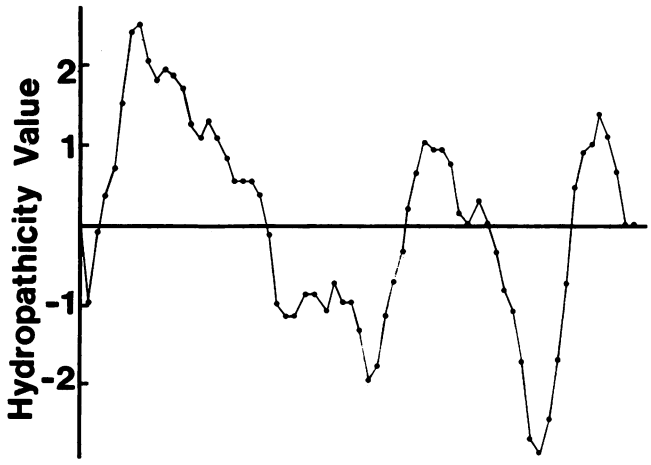

FIG. 5. Hydropathicity plot, calculated as described by Kyte and Doolittle (13), of the portion of OmpV fused to PhoA in (*ompV-phoA*)*2.2*(Hyb). This plot shows the hydrophobicity (points above the vertical axis) pattern typical of a signal sequence at the N terminus and indicates several additional domains of the mature protein that may be responsible for directing the OmpV portion to the outer membrane, from where the hybrid protein is released into the culture supernatant by *V. cholerae*.

The alkaline phosphatase activity of the RT110.21, a *tcpA-phoA* fusion isolate from strain O395, was assayed to determine its regulation and localization properties. As in the case of the *ctxA-phoA* fusion, the level of alkaline phosphatase activity reflected that of the gene product to which it was fused and was greatly reduced in the presence of the *toxR55* insertion mutation (Table 2), as expected since *tcpA* expression also requires a functional *toxR* gene product (32). On the other hand, the localization properties of the hybrid TcpA-PhoA protein were again not reflective of the native protein. Instead of being localized extracellularly, the hybrid remained cell associated, similar to what was seen for the PhoA fusion to the extracellular protein CtxB.

DISCUSSION

Fusions of alkaline phosphatase have proven useful in the analysis of extracytoplasmic proteins of *E. coli* (9, 10, 15, 16). We have extended this system through the construction of broad-host-range Tn*phoA* delivery vectors to allow the

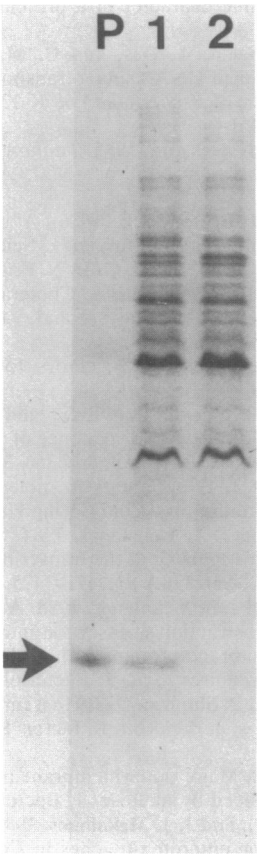

FIG. 6. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis total protein profiles of wild-type and *tcpA-phoA* fusion strains of *V. cholerae* O395. Lanes: P, TcpA pilin; 1, O395; 2, O395 Φ(*tcpA-phoA*)*21*(Hyb).

isolation of fusions in gram-negative species in addition to *E. coli*. These species should include those into which P-group plasmids or pBR322 can be introduced via conjugation or transformation and in which Tn*5* and its derivatives (Tn*phoA*) retain their ability to transpose. The isolation of active fusions has been successful even in such fastidious organisms as *Bordetella pertussis* with this system (11a).

We studied the properties of PhoA fusions in *V. cholerae* by utilizing fusions both to genes initially cloned on multicopy plasmids in *E. coli* and to chromosomal genes isolated directly in *V. cholerae*. Fusions to the cloned cholera toxin genes remained active when mobilized back into *V. cholerae*, and this activity was reflective of the expression of the gene to which they were fused. The utility of the broad-host-range delivery systems was demonstrated by the identification of fusions to a random gene expressing a secreted protein, *ompV*, as well as to a sought-after gene, *tcpA*, expressing a major pilus subunit. Restriction mapping and DNA sequence analysis of the points of Tn*phoA* insertion showed that in each active fusion isolated the junction occurred such that the alkaline phosphatase coding sequence was fused downstream of target gene sequences encoding secretion signals (i.e., hydrophobic N-terminal signal sequences or membrane-spanning segments). The insertions appear to occur relatively nonspecifically in *V. cholerae*, since we have been able to isolate fusions to several genes. To date, we have isolated over 10 independent Tn*phoA* fusions to *tcpA* as well as fusions to genes involved in pilus biosynthesis, toxin expression, motility, protease production, and other virulence properties of *V. cholerae* (R. Taylor, K. Peterson, S. Bortner, and J. Mekalanos, unpublished results).

Although *phoA* fusions appear to be useful in identifying genetic export signals in *V. cholerae*, as previously demonstrated in *E. coli* (15), results with the fusions studied here imply limited use with regard to the study of extracellular localization signals. The fusions to the cholera exotoxin subunits CtxA and CtxB were not found dissociated from the cell, despite the fact that the CtxB-PhoA fusion joint is only 8 amino acids from the carboxy terminus of CtxB. This could be because the fusions disrupt or delete protein domains required for this export or because the alkaline phosphatase moiety cannot readily be transported across the outer membrane by extracellular transport mechanisms. Interestingly, the fusion to OmpV, which is normally localized to the outer membrane, exhibits a significant amount of its activity extracellularly, suggesting that a PhoA fusion protein can on occasion reach the outside of a cell. It is possible that within the first 64 residues of OmpV there exists a domain for transport of the OmpV-PhoA fusion protein to the outer membrane of *V. cholerae*, but once so localized this hybrid protein is released from the cell because it lacks an OmpV C-terminal domain involved in anchoring it in the outer membrane. Such localization sequences have been demonstrated for several major outer membrane proteins from *E. coli* (1). It is interesting to speculate that such a mechanism may be utilized naturally by some gram-negative organisms for the extracellular secretion of certain proteins. Further studies are underway to determine whether the OmpV-PhoA hybrid protein retains sequences necessary for localization to the outer membrane and whether these domains can be defined by using a *phoA* fusion approach.

Perhaps the greatest benefit when using these vectors to isolate Tn*phoA* insertions in genetically diverse bacteria is the ability to enrich for defined mutations in genes that encode secreted proteins in the absence of any selection or previous knowledge as to their function. It is this property that makes the use of Tn*phoA* so valuable in the analysis of bacterial virulence. In this regard, the chromosomal fusion strains described here have been tested in vivo to assess the contribution of OmpV and TcpA in cholera pathogenesis (32). By comparing the mutants with the parent strains, with respect to their lethal dose and ability to compete for growth in an infant mouse model, it was shown that an *ompV-phoA* mutant strain and those containing random *phoA* fusions were not significantly decreased in these properties. In contrast, Tn*phoA* fusions in the structural gene (*tcpA*) for pilin protein yielded strains that showed a marked virulence defect and which were significantly outcompeted by the wild-type strain in vivo, demonstrating for the first time the role of a defined colonization factor in cholera pathogenesis (32).

The antibiotic resistance of Tn*phoA* provides a direct selection for cloning the insertion mutation generated upon fusion. This allows for the rapid analysis or characterization of the mutations by DNA sequencing and is the initial step in isolating the intact gene by providing a probe for hybridization screening of genomic banks. This allowed us to easily identify *ompV* and *tcpA* and has led to the more recent cloning and characterization of the intact *tcpA* gene (C. Shaw and R. Taylor, unpublished results).

The Tn*phoA* gene fusion approach also provides a simple and sensitive means of monitoring gene expression. This allowed us to demonstrate that *tcpA* expression is dependent on *toxR*, the positive regulatory gene controlling *ctxAB* expression (21). Thus, the use of Tn*phoA* offers a number of advantages

over other transposons in mutagenesis applications, particularly where secreted proteins are theoretical targets and where regulatory studies are an additional priority.

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