INFECTION AND IMMUNITY, Nov. 1988, p. 2822–2829
0019-9567/88/112822-08$02.00/0
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Vol. 56, No. 11

Characterization of the *Vibrio cholerae* ToxR Regulon: Identification of Novel Genes Involved in Intestinal Colonization

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Received 5 May 1988/Accepted 2 August 1988

A gene fusion library of *Vibrio cholerae* classical strain O395 was generated by using a broad host range vector for delivery of the transposon Tn*phoA*. The insertion library was screened for colonies expressing alkaline phosphatase-positive (PhoA$^+$) fusion proteins on LB agar at 30°C in the presence of 0.2% glucose. Over 600 PhoA$^+$ strains were isolated and then tested for regulation of their gene fusions in broth media that permitted high or low expression of cholera toxin. This strategy resulted in the isolation of 60 Tn*phoA* (Tn*5* IS*50*$_L$::*phoA*) fusions to genes encoding secreted proteins that are apparently coordinately regulated with cholera toxin. Introduction of a *toxR* null mutation into 10 of these fusion strains confirmed that these Tn*phoA* gene fusions are controlled either directly or indirectly by the cholera toxin transcriptional activator encoded by *toxR*. A combination of Southern and immunoblot analysis identified 17 distinct ToxR-regulated genes in *V. cholerae* O395. Many of these insertions were located in one of the two cholera toxin operon copies of strain O395, as well as a large gene cluster involved in the biogenesis of the toxin-coregulated pilus colonization factor. In addition, insertions were identified in genes that had no effect on either cholera toxin or toxin-coregulated pilus expression. Several of these insertions were localized to a cluster of four genes, the disruption of any of which by Tn*phoA* reduced the ability of strain O395 to colonize the intestines of suckling mice. The product encoded by this second gene cluster was named accessory colonization factor to describe its possible role in cholera pathogenesis. These studies reinforce the contribution of ToxR-regulated genes to the virulence properties of *V. cholerae*. This report also demonstrates a new approach for the identification of bacterial virulence factors, based on the characterization of genes that are regulated by the same environmental signals that control the expression of a known virulence factor.

Vibrio cholerae produces diarrheal disease in humans by colonizing the small bowel and secreting a proteinaceous enterotoxin (2, 13). The colonization of the intestinal mucosa by *V. cholerae* is a complex process. For example, it is thought that this organism expresses properties, such as motility and chemotaxis, to penetrate the mucous layer of the epithelium (5, 6); produces neuraminidase and protease, to degrade the mucus gel (1); produces surface pili and hemagglutinins, to adhere to microvilli (25); and produces toxins (enterotoxins and cytotoxins), to further enhance intraintestinal growth or survival (2, 13, 21). Because of the prominent role that cholera toxin plays in the secretory diarrhea seen in cholera, it has been the focus of intense genetic and biochemical investigation for over 20 years, whereas many of the other virulence properties of *V. cholerae* have received little attention. Only recently have the molecular components involved in the important process of intestinal colonization been studied by genetic and biochemical analysis. For example, the gene for the major subunit (*tcpA*) of the toxin-coregulated pilus (TCP) colonization factor of *V. cholerae* has been identified by an approach involving transposon mutagenesis with the vector Tn*phoA* (25). The Tn*phoA* vector facilitates the isolation of gene fusions between *phoA* (the gene for the *Escherichia coli* alkaline phosphatase) and target genes encoding secreted proteins (11) and is therefore very useful in the analysis of bacterial virulence determinants (25; R. K. Taylor, C. Manoil, and J. J. Mekalonos, J. Bacteriol., in press).

Another important observation made during these studies was that the expression of TcpA, an outer membrane protein called OmpU, and several other proteins is dependent on the product of the *toxR* gene that has been shown to be a transcriptional activator of the cholera toxin operon (*ctxAB*) (17–19). These data indicate the ToxR protein may regulate the transcription of multiple genes involved in the virulence of *V. cholerae*. There is a growing body of evidence indicating that many pathogenic bacteria regulate the expression of multiple virulence determinants in a coordinate fashion (3, 22–25, 28). These observations have prompted us to perform a more generalized search for other ToxR-regulated genes that may encode *V. cholerae* virulence determinants. Here we report the isolation and characterization of Tn*phoA* fusions to additional ToxR-regulated genes, most of which encode secreted proteins that enhance the colonization and virulence properties of *V. cholerae*.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and reagents. The bacterial strains and plasmids used in this study are shown in Table 1. Bacterial strains were stored at −70°C in Luria broth (LB) (16) with 20% glycerol. Tn*phoA* fusions were isolated after incubation at 30°C on LB agar containing 0.2% glucose and 40 μg of 5-bromo-4-chloro-3-indolyl phosphate (XP) per ml. To obtain maximum expression of ToxR-activated genes in broth cultures, cells were cultivated in LB at pH 6.5 at 30°C, M9 minimal broth (10) supplemented with 0.2% glycerol and 0.2% asparagine at 30°C, or 1% tryptone at pH 6.5 with 0.66 mM NaCl at 30°C as described previously

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description	Reference or source
Vibrio cholerae		
O395 Sm	Str^r	25
KP5.10[a]	*tcpA*::Tn*phoA* Str^r Kan^r	This study
KP9.79	*tcpA*::Tn*phoA* Str^r Kan^r	This study
KP8.88	*tcpA*::Tn*phoA* Str^r Kan^r	This study
KP8.97	*tcpB*::Tn*phoA* Str^r Kan^r	This study
KP2.21	*tcp*::Tn*phoA* Str^r Kan^r	This study
KP4.20	*tcp*::Tn*phoA* Str^r Kan^r	This study
KP5.20	*tcp*::Tn*phoA* Str^r Kan^r	This study
KP8.85	*tcp*::Tn*phoA* Str^r Kan^r	This study
KP8.96	*tcp*::Tn*phoA* Str^r Kan^r	This study
KP1.25	*ctxA*::Tn*phoA* Str^r Kan^r	This study
KP5.80	*ctx*::Tn*phoA* Str^r Kan^r	This study
KP9.62	*acfA*::Tn*phoA* Str^r Kan^r	This study
KP3.51	*acfB*::Tn*phoA* Str^r Kan^r	This study
KP3.44	*acfC*::Tn*phoA* Str^r Kan^r	This study
KP8.11	*acfD*::Tn*phoA* Str^r Kan^r	This study
KP2.16	?::Tn*phoA* Str^r Kan^r	This study
KP5.51	?::Tn*phoA* Str^r Kan^r	This study
KP8.56	?::Tn*phoA* Str^r Kan^r	This study
KP8.74	?::Tn*phoA* Str^r Kan^r	This study
KP1.25-12	*ctxA*::Tn*phoA* Str^r Kan^r Amp^r *toxR12*	This study
KP5.80-12	*ctx*::Tn*phoA* Str^r Kan^r Amp^r *toxR12*	This study
KP9.62-12	*acfA*::Tn*phoA* Str^r Kan^r Amp^r *toxR12*	This study
KP3.51-12	*acfB*::Tn*phoA* Str^r Kan^r Amp^r *toxR12*	This study
KP3.44-12	*acfC*::Tn*phoA* Str^r Kan^r Amp^r *toxR12*	This study
KP8.11-12	*acfD*::Tn*phoA* Str^r Kan^r Amp^r *toxR12*	This study
KP2.16-12	?::Tn*phoA* Str^r Kan^r Amp^r *toxR12*	This study
KP5.51-12	?::Tn*phoA* Str^r Kan^r Amp^r *toxR12*	This study
KP8.56-12	?::Tn*phoA* Str^r Kan^r Amp^r *toxR12*	This study
KP8.74-12	?::Tn*phoA* Str^r Kan^r Amp^r *toxR12*	This study
RT110.21	*tcpA*::Tn*phoA* Str^r Kan^r	25
Escherichia coli		
SM10(λpir)	*thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu (λpir)	25
MM294	*pro endA hsdR supF*	25
Plasmids		
pRT291	IncP Tra$^-$ Mob$^+$ Tc^r Km^r ::Tn*phoA*	25
pPH1JI	IncP Tra$^+$ Sp^r Gm^r Sm^r	25
pJM703.12	R6Kori RP4mob *toxR12* Ap^r	19

[a] Fusion strains are designated in the following manner: the numbers before a decimal point represent independent fusion pools, numbers following the decimal point are individual isolates within a given fusion pool, and the number 12 following a hyphen denotes the *toxR12* mutant allele (19).

(19). Antibiotics used in certain experiments included ampicillin (100 μg/ml), streptomycin (100 μg/ml), kanamycin (45 μg/ml), and gentamicin (30 μg/ml). Unless otherwise indicated, all chemical reagents were obtained from Sigma Chemical Co., St. Louis, Mo.

Tn*phoA* insertion library. Transposition of Tn*phoA* (11) into the genome of *V. cholerae* O395 Sm was performed as described elsewhere (25; Taylor et al., in press). Briefly, pRT291 (25; Taylor et al., in press), which contains Tn*phoA* in the mobilizing *E. coli* SM10, was transferred by conjugation into *V. cholerae* O395 Sm, and transconjugates were plated on medium containing streptomycin and kanamycin. Antibiotic-resistant colonies were collected, mated with *E. coli* MM294 carrying plasmid pPH1JI (25), and plated on medium containing gentamicin, kanamycin, streptomycin, and XP. Because pPH1JI is incompatible with pRT291, the antibiotic-resistant colonies obtained largely represented cells carrying Tn*phoA* insertions on the chromosome and pPH1JI. PhoA-positive colonies were picked and purified, and then cells were subsequently grown on medium without gentamicin in order to promote the loss of pPH1JI (Taylor et al., in press).

Nucleic acid preparation and analysis. Chromosomal DNA from *V. cholerae* was purified as described previously (12). Southern blot analysis was carried out under high-stringency conditions as described previously (12, 13). The 1,566-base-pair *Bam*HI-*Hin*dIII fragment derived from Tn*phoA* (corresponding to nucleotides 4973 to 6539 of Tn*phoA*) was used as a hybridization probe. This fragment (the TP-1 probe) was purified by electroelution from agarose gels and labeled with [^{32}P]dGTP by bacteriophage T4 DNA polymerase replacement synthesis (10). DNA restriction enzymes were purchased from New England BioLabs, Inc., Beverly, Mass.

SDS-PAGE and immunoblotting. Electrophoresis in 12.5% polyacryamide gels in the presence of sodium dodecyl sulfate (SDS-PAGE) was performed as described previously (9). Samples were prepared from 2 ml of cultures grown in LB at pH 6.5 in tubes (13 by 100 mm) incubated at 30°C for 18 to 24 h with constant mixing at 30 rpm on a roller incubator (model TC-7; New Brunswick Scientific Co., Inc., Edison, N.J.). The cells from 0.2 ml of culture were collected by centrifugation, suspended in 0.2 ml of SDS-PAGE sample buffer, placed in a boiling water bath for 5 min, and centrifuged for 5 min in an Eppendorf microcentrifuge. Samples (10 μl of the supernatant fluid) were loaded into each gel lane. After electrophoresis, gels were stained with Coomassie brilliant blue or proteins were transferred by electrophoresis to nitrocellulose sheets (27). The nitrocellu-

lose sheets were soaked in a Tris-buffered saline solution (TBS) (500 mM NaCl and 20 mM Tris hydrochloride [pH 7.4]) with 1% bovine serum albumin for 2 h at room temperature. Antibodies diluted 1:1,000 in TBS–5% nonfat dry milk (8) were then incubated with the nitrocellulose blots overnight at room temperature with gentle shaking. Following three washes in TBS for 15 min each, alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (ICN Biologicals, Costa Mesa, Calif.), diluted 1:2,000 in TBS–5% nonfat dry milk, was added and incubated for 3 h at room temperature. Following three washes in TBS, the blots were developed by the addition of 100 ml of 1 M Tris hydrochloride (pH 9.0) containing 100 μg of XP per ml and 500 μg of Nitro Blue Tetrazolium (previously dissolved in 5 ml of dimethylformamide) per ml.

Antisera. Antisera to purified *E. coli* alkaline phosphatase (Sigma) and TcpA pilin (25) were generated against stained protein bands eluted from gels after SDS-PAGE. New Zealand White rabbits were injected subcutaneously with a mixture of antigen and Freund complete adjuvant (Sigma), followed by booster injections 2 and 4 weeks later with antigen in Freund incomplete adjuvant. Rabbits were bled 14 days later, and serum was stored at 4°C until use.

Assay for alkaline phosphatase activity. Alkaline phosphatase (PhoA) activity was measured by determining the rate of hydrolysis of *p*-nitrophenyl phosphate by permeabilized cells (15). PhoA activity is expressed in Miller units (16). Cells were grown overnight as described previously (19), centrifuged, and washed in saline. Cells were added to 1 ml of 1 M Tris (pH 8.0), followed by the addition of 1 drop of 0.1% SDS and 2 drops of chloroform. The permeabilized cells were incubated at 37°C for 5 min prior to the addition of 100 μl of 0.4% *p*-nitrophenyl phosphate for color development at 37°C. The resulting PhoA activity was determined as previously described (25).

In vivo assay for *V. cholerae* intraintestinal growth. The competitive index for *V. cholerae* strains was determined in 3- to 5-day-old suckling CD1 mice inoculated orally as described previously (5, 25). Viable organisms were recovered by plating dilutions of intestinal homogenates on LB agar containing streptomycin and XP. The ratios of the recovered strains were scored for $PhoA^+$ (blue) and $PhoA^-$ (white) colonies representing Tn*phoA* insertion mutant and wild-type strains, respectively.

RESULTS

Isolation of Tn*phoA* fusions regulated by ToxR. A pool of over 10,000 random insertions of Tn*phoA* into the chromosome of classical *V. cholerae* Ogawa strain O395 was prepared by using the broad host range vector pRT291. Consistent with previous reports (25; Taylor et al., in press), approximately 1% of the colonies carrying Tn*phoA* inserts were blue on LB agar containing 0.2% glucose and 40 μg of XP per ml. A total of 600 blue colonies ($PhoA^+$) were purified and served as the starting bank of fusion strains. Our strategy for identifying ToxR-regulated gene fusions involved assaying these $PhoA^+$ colonies under physiologic growth parameters which were known to promote high or low expression of cholera toxin and the *tcpA* gene product (19, 25). The initial screen used cells grown at 30°C in LB at pH 8.4 ($ToxR^-$ condition) and LB at pH 6.5 ($ToxR^+$ condition). Approximately 130 clones exhibited at least a twofold variation in the level of *phoA* activity in these two media and were analyzed further. These strains were next cultivated at 30°C in 1% tryptone broth ($ToxR^-$ condition) and 1% tryptone containing 66 mM NaCl ($ToxR^+$ condition). Strains which continued to show differential activity in these two media were grown at 30°C in M9 glycerol minimal broth ($ToxR^-$ condition) and M9 glycerol broth supplemented with 0.2% asparagine ($ToxR^+$ condition). This screening process identified 60 strains which carried Tn*phoA* fusions to genes whose regulation was similar to that of *tcpA* and *ctxAB* in the six different media examined. We did not identify any gene fusions which appeared to be negatively regulated by ToxR (25) by this screening protocol. The PhoA activities of 17 presumptive ToxR-regulated fusions in these six media are presented in Table 2.

Genetic proof that these Tn*phoA* fusions were in ToxR-regulated genes was provided by introducing *toxR* null mutations into 10 of these strains. Plasmid pJM703.12, a suicide plasmid that contains a cloned internal restriction fragment of the *toxR* gene (19), was transferred into each strain by conjugation and integrated into the chromosomal *toxR* gene copy. Inactivation of the *toxR* gene in these fusion strains reduced PhoA activity in high-expression medium (LB at pH 6.5) to uninduced levels (Table 2). To produce a control, we also crossed pJM703.12 into several strains that produced higher PhoA activities in LB at pH 6.5 than in LB at pH 8.4 but did not show induction in either tryptone plus NaCl or M9 plus asparagine medium. In these strains, the elimination of functional *toxR* did not alter PhoA activity, suggesting that the pH regulation of these fusions was not mediated by ToxR (data not shown).

Identification of genes affecting pilus expression. It has been demonstrated in several bacterial systems that efficient assembly and expression of pili depends on multiple gene products (20). In *V. cholerae* O395, the corresponding genes for the TCP colonization factor (23) may be ToxR regulated. We therefore wanted to determine if the fusions we had identified affect TCP expression. Growth of *V. cholerae* O395 in LB at pH 6.5 at 30°C characteristically results in autoagglutination of cells, a property that is associated with TCP (25). Cultivation of the 60 ToxR-regulated fusion strains under these conditions identified 29 strains which did not autoagglutinate. In order to examine this phenotype in more detail, immunoblotting assays were performed with monospecific anti-TcpA antisera. Figure 1 illustrates the immunoblot profiles of representative fusion strains. Controls were wild-type O395 cells grown in LB at pH 6.5 (lane 5) and in LB at pH 8.4 (lane 4). Some fusion strains appeared to produce small amounts of immunodetectable TcpA (Fig. 1, lane 1), whereas others produced no detectable TcpA (Fig. 1, lane 2). All strains that displayed efficient autoagglutination (lane 3) produced wild-type levels of TcpA (lane 5). These data suggest that some of our *toxR*-regulated *phoA* fusions alter the ability of *V. cholerae* to express wild-type amounts of TcpA pilus subunits or to assemble these subunits on the cell surface. Recent work involving cosmid cloning, DNA sequencing, and additional transposon mutagenesis has extended these results (26) and has shown that a large gene cluster containing at least seven genes is involved in the biogenesis of the *V. cholerae* TCP colonization factor.

Physical mapping of ToxR-regulated Tn*phoA* gene fusions. A total of 31 of the ToxR-regulated Tn*phoA* gene fusions identified were to genes that did not alter TCP biogenesis. In order to determine how many unique genes these 31 *phoA* fusions represented, Southern blot analysis was performed. Chromosomal DNA from each strain was prepared and digested with *Xba*I, a restriction endonuclease that does not recognize Tn*phoA* sequences. A Tn*phoA* probe (TP-1) was then used to localize the inserts to specific *V. cholerae Xba*I

TABLE 2. Alkaline phosphatase activity[a] of ToxR-regulated gene fusions

Strain (fusion)[b]	Culture conditions[c]					
	LB		1% Tryptone		M9	
	pH 6.5	pH 8.4	With 66 mM NaCl	Without NaCl	With 0.2% Asn	Without 0.2% Asn
KP1.25 (*ctxA*)	810	58	517	63	544	58
KP1.25-12	41	50	ND	ND	ND	ND
KP5.80 (*ctx*)	460	41	324	23	307	24
KP5.80-12	36	45	ND	ND	ND	ND
KP9.62 (*acfA*)	990	43	520	80	508	63
KP9.62-12	26	30	ND	ND	ND	ND
KP3.51 (*acfB*)	137	6	77	8	71	9
KP3.51-12	12	10	ND	ND	ND	ND
KP3.44 (*acfC*)	324	14	187	20	196	18
KP3.44-12	24	28	ND	ND	ND	ND
KP8.11 (*acfD*)	210	11	130	14	136	13
KP8.11-12	18	14	ND	ND	ND	ND
KP2.16 (?)	14	2	8	2	9	3
KP2.16-12	3	3	ND	ND	ND	ND
KP5.51 (?)	84	4	51	6	56	6
KP5.51-12	8	6	ND	ND	ND	ND
KP8.56 (?)	180	6	108	8	120	9
KP8.56-12	12	14	ND	ND	ND	ND
KP8.74 (?)	62	3	41	6	36	7
KP8.74-12	6	4	ND	ND	ND	ND
KP8.96 (*tcpA*)	110	67	66	47	60	54
KP9.79 (*tcpB*)	960	95	600	54	660	58
KP8.97 (*tcp*)	37	8	31	5	34	6
KP5.20 (*tcp*)	48	8	38	4	41	5
KP8.85 (*tcp*)	16	3	10	3	12	3
KP4.20 (*tcp*)	31	4	22	5	24	4
KP2.21 (*tcp*)	95	20	71	14	70	10

[a] As described in Materials and Methods.
[b] See Table 1.
[c] Cells were grown overnight at 30°C in the various media, which are described in Materials and Methods. ND, Not determined.

fragments. These studies identified seven unique chromosomal fragments containing Tn*phoA* insertions (Fig. 2, lanes A through G). Two of these fragments (Fig. 2, lanes B and C) were identified by additional Southern blot analysis as the two *Xba*I fragments that carry one copy each of the *ctxAB* operon of strain O395 (14) (data not shown). The smaller lighter band seen in lane B represents homogenization of *ctx*::Tn*phoA* into the second copy of the *ctx* operon via homologous recombination (14). A total of 11 strains carried Tn*phoA* fusions in one or the other of these two *Xba*I

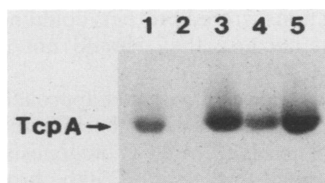

FIG. 1. Immunoblot analysis of the TCP pilin (TcpA) present in total *V. cholerae* protein preparations. Following SDS-PAGE, proteins were transferred to nitrocellulose and probed with monospecific antisera to TcpA. Lanes: 1, KP8.95; 2, KP9.79; 3, KP1.25; 4 and 5, O395 parental strain. All samples were prepared from cells grown in LB at pH 6.5 at 30°C, except for lane 4 (cells grown in LB at pH 8.4). The immunoblot profile in lane 1 was identical to those seen in other ToxR-regulated Tn*phoA* fusion strains that no longer autoagglutinate in LB at pH 6.5 but do not carry inserts in *tcpA*. Lane 2, Representative *tcpA*::Tn*phoA* fusion strain. Lane 3, Representative strain carrying a ToxR-regulated Tn*phoA* fusion that does not affect TCP expression or autoagglutination.

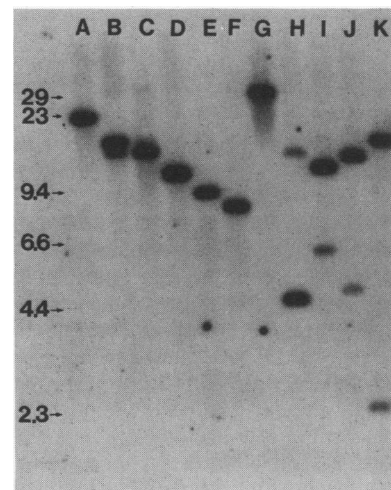

FIG. 2. Southern blot analysis of strains carrying ToxR-regulated Tn*phoA* insertions. Chromosomal DNA for the indicated strains was digested with *Xba*I (lanes A through G) or *Xba*I plus *Bam*HI (lanes H through K), electrophoresed, transferred to nitrocellulose, and hybridized to the TP-1 probe. Lanes: A, KP2.16; B, KP1.25; C, KP5.80; D, KP5.51; E, KP8.74, F, KP8.56; G, KP3.51; H, KP9.62; I, KP3.51; J, KP3.44; K, KP8.11. DNA from strains KP9.62, KP3.44, and KP8.11 gave *Xba*I fragments in this analysis that were identical to that seen in lane G. Numbered arrows on the left show the sizes in kilobases and positions of *Hin*dIII-digested lambda phage DNA fragments.

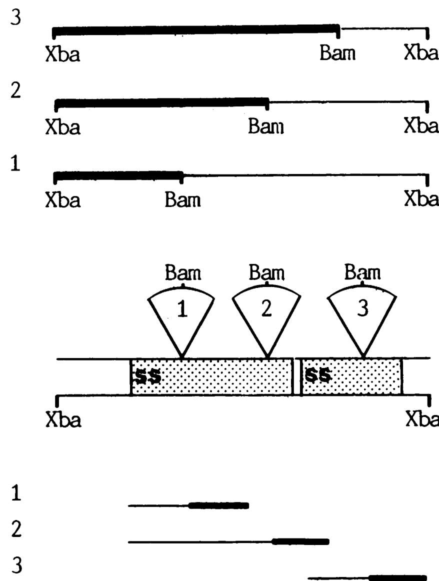

FIG. 3. Schematic diagram of the strategy used to physically map unique genes carrying ToxR-regulated Tn*phoA* insertions. A single *XbaI* chromosomal fragment of DNA (in which three different Tn*phoA* inserts have been localized) is depicted in the center of the diagram. In this example, the fragment carries two genes (▭) encoding proteins with signal sequences (SS) and the three independent Tn*phoA* inserts (numbered pie slices) are fused to these two genes in the same orientation as indicated. The three lines at the top of the illustration depict fragments detected in a Southern blot analysis of chromosomal DNA digested with *XbaI* plus *BamHI* and hybridized to the TP-1 probe (a Tn*phoA* probe that recognizes DNA flanking both sides of the *BamHI* site). The lines to the left of the *BamHI* sites are thicker to illustrate the fact that the TP-1 probe contains more homology to these upstream junction fragments containing *phoA* sequences and thus produces a darker autoradiographic band. The three lines at the bottom represent expected PhoA fusion proteins detectable by immunoblot with anti-PhoA antibodies. The thicker portion of these lines represents PhoA polypeptide, while the total lengths of the lines correspond to the expected size of the fusion protein.

fragments. Thus, the remaining 20 strains have Tn*phoA* fusions to ToxR-regulated genes other than those involved in either cholera toxin or TCP production.

Interestingly, 10 of these strains contained inserts that were localized to a single 29-kilobase (kb) *XbaI* fragment (Fig. 2, lane G). In order to determine if this fragment contained Tn*phoA* insertions in the same gene or in multiple linked genes, additional Southern blot and immunoblot analyses were performed. We utilized the TP-1 probe (which recognizes sequences on both sides of the single *BamHI* cleavage site of Tn*phoA* but shows more homology to sequences upstream of this site) in Southern blot analyses of *XbaI*-plus-*BamHI* double digests to establish a tentative linear order of these fusions along the chromosome. We then used anti-PhoA antibodies in an immunoblot assay to correlate this order with the size of PhoA fusion proteins.

Figure 3 schematically represents the strategy used in these experiments for a theoretical example involving three independent Tn*phoA* fusions in a single *XbaI* fragment containing two distinct genes encoding secreted proteins. Following Southern hybridization, two bands would be seen. The darker band would be the upstream *XbaI*-*BamHI* fragment containing the *phoA* coding sequence and fusion junction, while the lighter band would be the fragment carrying

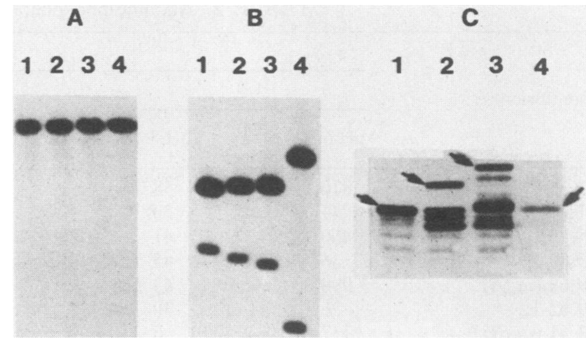

FIG. 4. Physical mapping of Tn*phoA* fusions affecting TCP expression. Panels A and B show Southern blot analysis of chromosomal DNA from the indicated strains digested with either *XbaI* (A) or *XbaI* plus *BamHI* (B) and then hybridized to the TP-1 probe. Panel C shows results from an immunoblot analysis of PhoA fusion proteins produced by the indicated strains. Arrows denote full-length fusion proteins detected with anti-PhoA antibodies. Lanes: 1, KP9.79; 2, KP8.88; 3, KP5.10; 4, KP8.97.

sequences downstream of the *BamHI* site. Assuming that all genes present on the *XbaI* fragment are in the same orientation (as would be the usual case for an operon) and that the only *BamHI* site is within Tn*phoA*, the size of the upstream *XbaI*-*BamHI* fragments allows one to deduce the linear order of the fusion series. By then performing immunoblot analysis using anti-PhoA antisera, one can determine the sizes of the hybrid proteins produced by each Tn*phoA* gene fusion and correlate these sizes with those of the corresponding *XbaI*-*BamHI* fragments. In the case where two strains carry different insertions in the same gene, an increase in the size of the strongly hybridizing *XbaI*-*BamHI* fragment should correlate with production of a PhoA hybrid protein with an appropriate increase in apparent molecular weight (Fig. 3, inserts 1 and 2). In the case where two strains carry different inserts in two adjacent genes (Fig. 3, inserts 2 and 3), the strain containing Tn*phoA* fused to the downstream gene (i.e., the one showing the larger *Xba*-*BamHI* upstream junction fragment) will usually not produce a fusion protein whose size correlates exactly with the increased size of the corresponding *XbaI*-*BamHI* fragment seen in the Southern analysis. We assume that 1 kb of DNA has coding capacity for approximately 36 kilodaltons of protein. The correct interpretation of these results depends on the availability of a collection of closely spaced Tn*phoA* insertions that encode fusion proteins of reasonable stability. Genes encoding nonsecreted proteins could not be picked up in this analysis, because they should not encode $PhoA^+$ hybrid proteins (11).

Figure 4 shows the results of this approach when applied to our Tn*phoA* fusions known to be inserted in two genes (*tcpA* and *tcpB*) involved in TCP biogenesis (26). All four fusions were mapped to the same 23-kb chromosomal *XbaI* fragment by Southern analysis (Fig. 4A). Double digests with *XbaI* and *BamHI* (Fig. 4B) allowed the four strains to be ordered such that the strongly hybridizing bands increase in size and thus provide the physical order of the gene fusion series. Finally, immunoblot analysis of the PhoA hybrid proteins produced by the four strains allowed us to tentatively conclude that the first three fusion strains (lanes 1 through 3) carry Tn*phoA* fusions to the same gene (*tcpA*), while the strain in lane 4 carries a Tn*phoA* fusion to a different downstream gene (*tcpB*). The lower-molecular-weight immunoreactive bands probably represent PhoA fu-

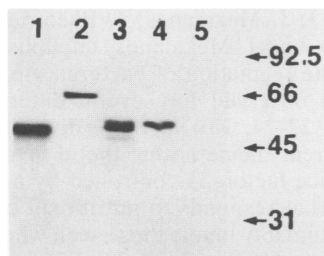

FIG. 5. Immunoblot analysis of PhoA fusion proteins produced by *acf* mutants and the parental strain. Strains were grown in LB at pH 6.5 at 30°C and analyzed with anti-PhoA antibody. Lanes: 1, KP9.62; 2, KP3.51; 3, KP3.44; 4, KP8.11; 5, O395 (parental strain). Numbers on the right denote molecular masses in kilodaltons of Bio-Rad low-molecular-mass standard proteins.

sion protein degradative products (11). The intensity of staining of the fusion band for the *tcpB* fusion (Fig. 4, lane 4) also correlates with the assignment of this new gene, which apparently is not expressed as well as *tcpA* at either the translational or transcriptional level.

This physical mapping approach was applied to the four Tn*phoA* fusion strains that do not show any alteration in TCP or cholera toxin expression and which also carry insertions in apparently the same 29-kb *Xba*I fragment (Fig. 2, lane G). Double digestion using *Xba*I plus *Bam*HI of DNA from these four strains produced fragments that strongly hybridized to the TP-1 probe that were 4.7 (KP9.62), 10.8 (KP3.51), 12.1 (KP3.44), and 14.4 (KP8.11) kb in size (Fig. 2, lanes H through K). Immunoblot analysis (Fig. 5) showed these four strains to produce PhoA hybrid proteins of 49 (KP9.62), 63 (KP3.51), 53 (KP3.44), and 53 kilodaltons (KP8.11). The fusion protein produced by strain KP3.44 appears to be relatively unstable (11), since we detect three immunoreactive proteins: 53, 51, and 49 kilodaltons. Because the sizes of the PhoA fusion proteins do not correlate with the physical order and linear separation of the four corresponding Tn*phoA* insertions, these data suggest that these four Tn*phoA* fusions define four distinct ToxR-regulated genes on this 29-kb *Xba*I fragment. In addition, the different *phoA* activities of these four strains (Table 2) reinforce this conclusion. Of course, proof of this interpretation will require the cloning and sequencing of these Tn*phoA* fusions. We have tentatively designated the four genes identified by this analysis *acfA*, *acfB*, *acfC*, and *acfD* (for accessory colonization factor; see below).

Mouse colonization assays. Since mutations in the three ToxR-regulated genes identified to date (*ctxA*, *ctxB*, and *tcpA*) dramatically reduce the virulence of *V. cholerae*, we were interested in testing all of our strains carrying ToxR-regulated Tn*phoA* gene fusions for colonization and virulence defects. We first used a competition assay for colonization in which the parental O395 strain was coinfected with individual fusion strains into 3- to 5-day-old infant CD1 mice as described previously (25). Table 3 summarizes the ability of strains carrying inserts in 17 different ToxR-regulated genes to colonize the intestines of suckling mice as measured by this assay. As expected, insertions in the genes that affected TcpA-mediated autoagglutination dramatically reduced the ability of these fusion strains to compete with the parental strain. Tn*phoA* insertions in either of the two *ctxAB* operons also caused slightly decreased colonization in this assay. Strains KP5.51, KP8.56, and KP8.74, each carrying a ToxR-regulated Tn*phoA* fusion, competed very effectively with the parental O395 strain (Table 3). Mutants KP9.62,

TABLE 3. Identification of colonization defects in strains containing *phoA* gene fusions to ToxR-regulated genes

Strain[a]	Fusion	Competitive index[b]	
		In vitro	In vivo
KP9.79	*tcpA*::Tn*phoA*	1.03	<0.002
KP8.97	*tcpB*::Tn*phoA*	1.14	<0.002
KP8.96	*tcp*::Tn*phoA*	1.40	<0.002
KP5.20	*tcp*::Tn*phoA*	1.06	<0.002
KP8.85	*tcp*::Tn*phoA*	1.20	<0.002
KP4.20	*tcp*::Tn*phoA*	1.30	<0.002
KP2.21	*tcp*::Tn*phoA*	1.20	<0.002
RT110.21	*tcpA*::Tn*phoA*	1.16	<0.002
KP1.25	*ctxA*::Tn*phoA*	0.87	0.36
KP5.80	*ctx*::Tn*phoA*	1.05	0.40
KP5.51	?::Tn*phoA*	1.24	1.10
KP8.56	?::Tn*phoA*	0.97	0.88
KP8.74	?::Tn*phoA*	0.92	0.93
KP9.62	*acfA*::Tn*phoA*	0.91	0.06
KP3.51	*acfB*::Tn*phoA*	0.93	0.04
KP3.44	*acfC*::Tn*phoA*	0.99	0.07
KP8.11	*acfD*::Tn*phoA*	1.08	0.02
KP2.16	?::Tn*phoA*	0.78	0.38

[a] See Table 1.

[b] Competitive index is defined as the change in the ratio of two strains after growth together under experimental conditions. The in vitro condition was growth at 30°C for 18 h in LB at pH 6.5. The in vivo condition was as described in Materials and Methods. In vivo competitive indices are the averages of values obtained from 12 to 15 individual mouse experiments.

KP3.51, KP3.44, and KP8.11, which carry closely linked Tn*phoA* inserts in *acf* genes (Fig. 2 and 5), showed a pronounced colonization defect that was similar in magnitude for each strain. Consistent with this result, we observed a 10-fold increase in the 50% lethal doses of these four *acf* mutant strains in suckling mice (data not shown). These data indicate that virulence or colonization defects can be detected in most derivatives of strain O395 that carry Tn*phoA* insertions in ToxR-regulated genes.

DISCUSSION

Recent studies (19, 25) demonstrating the coordinate regulation of two major *V. cholerae* virulence factors (cholera toxin and TCP) by ToxR prompted us to perform a generalized search for other ToxR-regulated genes. The transposon Tn*phoA* was used to generate protein fusions between PhoA and secreted proteins of *V. cholerae*. Since expression of cholera toxin and TCP are regulated by the same physiological parameters, we predicted that other ToxR-regulated gene products might show a similar pattern of expression (Table 2). This strategy led to the identification of 60 ToxR-regulated Tn*phoA* gene fusions. Chromosomal structural analysis of the Tn*phoA* insertions, together with physical characterization of PhoA fusion proteins they encoded, indicated the existence of at least 17 distinct ToxR-regulated genes represented by the following fusion strains: KP2.16, KP5.51, KP8.56, KP8.74, KP5.10, KP8.97, KP2.21, KP4.20, KP5.20, KP8.85, KP8.96, KP1.25, KP5.80, KP9.62, KP3.44, KP3.51, and KP8.11 (Table 2 and Fig. 2) (26).

Many of the Tn*phoA* insertions were localized to genes involved in TCP and cholera toxin expression. We also hoped to isolate fusions to OmpU and OmpT, two outer membrane proteins which appear to be regulated by ToxR (19, 25), but such fusions were not identified. Our success in isolating Tn*phoA* fusions to other ToxR-regulated genes suggests that insertion mutations in *ompU* or *ompT* may be

lethal events or that OmpU and OmpT expression is not exclusively regulated by ToxR (19).

Another class of genes we expected to identify in our analysis of ToxR-regulated Tn*phoA* fusions were those involved in TCP biogenesis. This was anticipated because in other systems, such as the Pap pilus of *E. coli* (20), the assembly of functional pili on the cell surface requires the participation of a set of coordinately expressed gene products, all of which are secreted through the bacterial cytoplasmic membrane. Analogous genes involved in TCP biogenesis in *V. cholerae* were therefore likely to exist, to be regulated by ToxR, and to yield $PhoA^+$ hybrid proteins on fusion to Tn*phoA* (11). Indeed, about half of the 60 fusions we isolated failed to produce the characteristic TCP-mediated autoagglutination when cultured in LB at pH 6.5 at 30°C. The importance of these gene products for TCP expression is clearly demonstrated by the dramatic colonization defect exhibited by each of these fusion strains (Table 3). The identification of these TCP accessory genes should help in dissecting the mechanism of assembly, the structure, and the adhesin properties of this *V. cholerae* pilus colonization factor (26).

In addition to the *ctx* structural genes and TCP biogenesis genes, we were able to identify *phoA* fusions to ToxR-regulated genes that do not appear to affect cholera toxin or TCP expression. Interestingly, 10 of these latter fusions were localized to the same 29-kb *Xba*I chromosomal fragment. A more detailed analysis employing a combination of Southern blot and immunoblot analyses indicated that this *Xba*I fragment carries a cluster of at least four genes (*acfA*, *acfB*, *acfC*, and *acfD*) encoding secreted proteins.

The molecular basis for reduced virulence in *V. cholerae acf*::Tn*phoA* mutants in suckling mice is not known. Because these genes are closely linked and insertion mutations in any of them produced quantitatively the same colonization and virulence defect, we would predict that these genes work together to alter a given product of *V. cholerae*. We have tentatively named this product accessory colonization factor or ACF to distinguish its phenotype from TCP. ACF might represent a new *V. cholerae* pilus (distinct from TCP) (7) or a nutrient-scavenging pathway. Given the complex nature of the intestinal mucosal environment, it is not surprising that *V. cholerae* would utilize multiple products to enhance the colonization process.

These studies also introduce a new strategy for the identification of bacterial virulence factors. By isolation of gene fusions whose expression is controlled by the same physiological factors that control the expression of a known virulence determinant, we have shown that mutations in genes encoding additional virulence determinants can be readily isolated. In the example reported here, Tn*phoA* insertions in genes encoding ACF and TCP were identified as fusions that were regulated by the same physiological signals that regulated cholera toxin (19, 25). Fully 83% (50 of a total of 60) of our toxin-coregulated Tn*phoA* fusions turned out to be insertions in genes whose inactivation reduced the colonization and virulence properties of *V. cholerae*. The success of this approach has, as its molecular basis, the fact that the same regulatory protein (ToxR) apparently controls the expression of cholera toxin and many other genes encoding secreted virulence determinants of *V. cholerae* (19, 25). In an analogous approach, we have recently characterized a bank of Tn*phoA* fusions in *Bordetella pertussis* that has led to a similar dramatic enrichment of mutations that affect coordinately regulated virulence properties of this organism (S. Knapp and J. J. Mekalanos, J. Bacteriol., in press; T. Finn, S. Knapp, and J. Mekalanos, unpublished results).

The coordinate regulation of bacterial virulence determinants has been observed for several different pathogenic bacteria (4, 18, 22–24, 28). In these diverse bacterial systems, the recurrent theme is that the in vivo expression of multiple virulence factors is controlled by a central regulatory protein(s) that responds to nutritional or physiological signals that presumably mimic those seen when the pathogen enters host tissues (4, 18, 22–24, 28). This coordinate regulation reinforces the fact that virulence is a multifaceted property involving the cooperative interaction of many different proteins, each of which contributes to the survival of the microbe within the host and thus the appearance of disease.

The results presented here indicate that the growth of *V. cholerae* under conditions that activate the ToxR regulon will result in the expression of a large set of secreted proteins, many of which contribute to the virulence of *V. cholerae*. These proteins represent potential targets for the immune system and their presence in cholera vaccines made up of dead whole cells may improve the efficacy of this type of cholera vaccine (19, 25, 26). Furthermore, we would also predict that serological responses to some ToxR-regulated proteins might also be the best means of assessing immunity to *V. cholerae*. Thus, identification and characterization of coordinately regulated virulence determinants should lead to a more thorough understanding of microbial pathogenesis and thus provide new strategies for immunoprophylaxis, diagnosis, and control of infectious disease.

ACKNOWLEDGMENTS

We thank R. K. Taylor and V. J. DiRita for their helpful comments.

This work was supported by Public Health Service grants AI-18045 and AI-26289 from the National Institute of Allergy and Infectious Diseases. K.M.P. was supported by National Research Service award AI-07458-02 from the National Institutes of Health.

LITERATURE CITED

1. **Baselski, V. S., R. A. Medina, and C. D. Parker.** 1979. In vivo and in vitro characterization of virulence-deficient mutants of *Vibrio cholerae*. Infect. Immun. **24:**111–116.
2. **Betley, M. J., V. L. Miller, and J. J. Mekalanos.** 1986. Genetics of bacterial enterotoxins. Annu. Rev. Microbiol. **40:**577–605.
3. **Calderwood, S. B., F. Auclair, J. J. Mekalanos, and G. T. Keusch.** 1987. Nucleotide sequence of the iron-regulated shiga-like toxin genes of *Escherichia coli*. Proc. Natl. Acad. Sci. USA **84:**4364–4368.
4. **Calderwood, S. B., and J. J. Mekalanos.** 1987. Iron regulation of Shiga-like toxin expression in *Escherichia coli* is mediated by the *fur* locus. J. Bacteriol. **169:**4759–4764.
5. **Freter, R., P. C. M. O'Brien, and M. M. S. Macsai.** 1981. Role of chemotaxis in the association of motile bacteria with intestinal mucosa: in vivo studies. Infect. Immun. **34:**234–240.
6. **Guenzel, M. N., and L. J. Berry.** 1975. Motility as a virulence factor of *Vibrio cholerae*. Infect. Immun. **15:**539–548.
7. **Hall, R. H., P. A. Vial, J. B. Kaper, J. J. Mekalanos, and M. M. Levine.** 1988. Morphological studies on fimbriae expressed by *Vibrio cholerae* O1. Microb. Pathogen. **4:**257–265.
8. **Johnson, D. A., J. W. Gautsch, J. R. Sportsman, and J. J. Eider.** 1984. Improved technique utilizing nonfat dry milk for analysis of proteins and nucleic acids transferred to nitrocellulose. Gene Anal. Tech. **1:**3–8.
9. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
10. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory,

Cold Spring Harbor, N.Y.

11. **Manoil, C., and J. Beckwith.** 1985. Tn*phoA*: a transposon probe for protein export signals. Proc. Natl. Acad. Sci. USA **82**:8129–8133.
12. **Mekalanos, J. J.** 1983. Duplication and amplification of toxin genes in *Vibrio cholerae*. Cell **35**:253–263.
13. **Mekalanos, J. J.** 1985. Cholera toxin: genetic analysis, regulation, and role in pathogenesis. Curr. Top. Microbiol. Immunol. **118**:97–118.
14. **Mekalanos, J. J., D. J. Swartz, G. D. N. Pearson, N. Harford, F. Groyne, and M. deWilde.** 1983. Cholera toxin genes: nucleotide sequence, deletion analysis and vaccine development. Nature (London) **306**:551–557.
15. **Michaelis, S. H., D. Inouye, D. Oliver, and J. Beckwith.** 1979. Mutations that alter the signal sequence of alkaline phosphatase in *Escherichia coli*. J. Bacteriol. **154**:366–374.
16. **Miller, J. H.** 1972. Molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
17. **Miller, V. L., and J. J. Mekalanos.** 1984. Synthesis of cholera toxin is positively regulated at the transcriptional level by *toxR*. Proc. Natl. Acad. Sci. USA **81**:3471–3475.
18. **Miller, V. L., R. K. Taylor, and J. J. Mekalanos.** 1987. Cholera toxin transcriptional activator ToxR is a transmembrane DNA binding protein. Cell **48**:271–279.
19. **Miller, V. L., and J. J. Mekalanos.** 1988. A novel suicide vector and its use in construction of insertion mutations: osmoregulation in *Vibrio cholerae* of outer membrane proteins and virulence determinants require *toxR*. J. Bacteriol. **170**:2575–2583.
20. **Normark, S., M. Baga, M. Goransson, F. P. Lindberg, B. Lund, M. Norgren, and B. E. Uhlin.** 1986. Genetics and biogenesis of *Escherichia coli* adhesins, p. 113–143. *In* D. Mirelman (ed.), Microbial lectins and agglutinins. John Wiley & Sons, Inc., New York.
21. **Pierce, N. F., J. B. Kaper, J. J. Mekalanos, and W. C. Cray.** 1985. Role of cholera toxin in enteric colonization of *Vibrio cholerae* O1 in rabbits. Infect. Immun. **50**:813–816.
22. **Recsei, P., B. Kreiswirth, M. O'Reilly, P. Schlievert, A. Gruss, and R. P. Novick.** 1986. Regulation of exoprotein genes in *Staphylococcus aureus* by *agr*. Mol. Gen. Genet. **202**:58–61.
23. **Stachel, S. E., and P. C. Zambryski.** 1986. *virA* and *virG* control plant-induced activation of the T-DNA transfer process of *A. tumefaciens*. Cell **46**:325–333.
24. **Straley, S. C., and W. S. Bowmer.** 1986. Virulence genes regulated at the transcriptional level by Ca^{2+} in *Yersinia pestis* include structural genes for outer membrane proteins. Infect. Immun. **51**:445–454.
25. **Taylor, R. K., V. L. Miller, D. B. Furlong, and J. J. Mekalanos.** 1987. Use of *phoA* gene fusions to identify a pilus colonization factor coordinately regulated with cholera toxin. Proc. Natl. Acad. Sci. USA **84**:2833–2837.
26. **Taylor, R. K., C. Shaw, K. M. Peterson, P. Spears, and J. J. Mekalanos.** 1988. Safe, live *Vibrio cholerae* vaccines? Vaccine **6**:151–154.
27. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins. Proc. Natl. Acad. Sci. USA **76**:4350–4354.
28. **Weiss, A. A., and S. Falkow.** 1984. Genetic analysis of phase change in *Bordetella pertusis*. Infect. Immun. **43**:263–269.