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Plasmid-Mediated Changes in Virulence of *Vibrio cholerae*

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The effects of several plasmids, including cloning vectors and R factors, on the virulence of *Vibrio cholerae* CA401R were determined by measuring the dose-related diarrheal response in orally challenged infant mice. The plasmids were also examined for their effects on the colonization ability of strain CA401R by joint infection experiments with a spectinomycin-resistant CA401 strain as an internal standard. One *V. cholerae* R factor, pVH2, enhanced the diarrheal response, while R factors Rts1 and pVH1 reduced it; plasmids RP4, pRK290, Sa, pSJ8, pSJ5, and pBR328 had no effect. The ability of the plasmids to affect in vitro toxin production by CA401R was variable. Cells containing large plasmids all showed a modest decrease in colonization ability. These results showed that some plasmids affected *V. cholerae* virulence, but that the cloning vectors pBR328, RP4, and pRK290 did not.

Plasmids may reduce the virulence of their bacterial hosts. Plasmid Sa, originally isolated from *Shigella flexneri* (22), reduces the virulence of *Agrobacterium tumefaciens* in plants (17). Sinha and Srivastiva (39) found that two conjugative plasmids (P, the *Vibrio cholerae* sex factor [6], and V [7]) reduced the virulence of *V. cholerae*. However, plasmids often enhance the pathogenicity of their host bacteria (15). Such virulence-associated plasmids may carry toxin genes (40), code for adhesins (16), or contain other genes that affect bacterial virulence (24, 37). Most virulence-associated plasmids have been found in virulent bacteria. Two drug resistance plasmids used in this study, pVH1 and pVH2, were present in virulent *V. cholerae* strains isolated during a cholera outbreak in Bangladesh (44).

To study the pathogenic mechanisms of *V. cholerae* by using recombinant DNA techniques, suitable cloning vectors that can be introduced and maintained stably are required. The in vivo virulence of *V. cholerae* should be unaffected by the presence of the cloning vectors. Plasmid pRK290 of the P incompatibility group has been used as a cloning vector for *V. cholerae* (26, 41). The conjugative plasmid Rp4 (11), of the same incompatibility group as pRK290, has also been used in cloning studies (9, 13) and can be maintained stably in *V. cholerae*. Another commonly used cloning vector, pBR328, was used to introduce *V. cholerae* genes into *Escherichia coli* (31). We want to be able to assess virulence in an animal model by using *V. cholerae* strains containing cloned DNA. Therefore, we examined these potential cloning vectors for their effects on virulence.

We began by introducing each of the following plasmids into *V. cholerae* CA401R: pSJ5 and pSJ8 (*V. cholerae* sex factor P derivatives marked with Tn*1* and Tn*9* insertions [23]), pVH1; pVH2; pRK290; Rp4; and pBR328. We tested CA401R containing each plasmid for (i) the ability to induce a fluid accumulation (FA) response in infant mice (2, 45); (ii) the ability to produce cholera toxin (CT), as measured by the Y1 adrenal cell assay (36); and (iii) the ability to colonize the upper part of the small intestine of infant mice, as evaluated by dual infection competition experiments (34). Two other plasmids which are maintained stably in *V. cholerae*, Rts1, the temperature-sensitive R plasmid originally isolated in *Proteus vulgaris* (10, 47), and Sa (22), were included in the study.

We found that plasmid PVH2 enhanced the virulence of *V. cholerae* CA401R, while plasmids PVH1 and Rts1 reduced it. Cloning vectors PRK290, RP4, and pBR328 had no effect on *V. cholerae* virulence.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are listed in Table 1. Conjugative plasmids were introduced into *V. cholerae* CA401R from *V. cholerae* or *E. coli* donors either by the broth mating or by the plate mating technique (32). The following bacterial strains were donors of their respective plasmids: *E. coli* J53 was donor of Sa and Rp4; *E. coli* 711 was donor of Rts1; *V. cholerae* MT6968 was donor of pVH1; and *V. cholerae* MT5726 was donor of pVH2. Plasmid pRK290 was mobilized from *E. coli* HB101 to *V. cholerae* CA401 by the triparental mating technique (35), with plasmid pRK2013 as a helper.

Plasmid pBR328 was introduced into CA401 by transformation. Briefly, *V. cholerae* cells that had been grown to a density of about 10^8 cells per ml were washed with a solution of 100 mM Tris (pH 8.0) and suspended in Tris-EDTA solution (10 mM Tris, 1 mM EDTA, pH 8.0). Cells were made competent by the addition of 40% (wt/vol) polyethylene glycol solution and mixed with plasmid DNA.

Media. Brain heart infusion broth (BHI; Difco Laboratories, Detroit, Mich.) and meat extract agar (MEA) were used as described previously (2). Antibiotics were added to selective media at the following concentrations (in micrograms per milliliter): rifampin, 80; spectinomycin, 60; kanamycin and carbenicillin, 40; chloramphenicol, 30; tetracycline, 15 (for *E. coli*), and 5 (for *V. cholerae*); and trimethoprim, 100. Strain CA401R(pBR328) was maintained on selective antibiotic media owing to the high frequency of segregation of pBR328.

Hemolytic activity. The strains were plated on BHI agar supplemented with 5% sheep blood and examined after 24 and 48 h of incubation at 37°C.

Determination of diarrheal response. The dose-related diarrheal responses in suckling mice were measured as previously described (2), with slight modifications. Briefly, strain CA401R with or without plasmids was grown for 18 to 20 h on BHI slants and suspended in BHI broth containing 0.01% Evans blue dye (Eastman Kodak Co., Rochester, N.Y.).

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant phenotype[a]	Incompatibility group	Reference	Source
Strain				
CA401	Vir		2,3	
CA401R	Vir, *rif*-100[b]			This study
CA401S	Vir, *spc*-110[b]			This study
VB-12	Rough mutant of CA401		4	
569B	Toxin overproducer		4	
FA9	Hemolysin-deficient mutant of CA401		5	
Plasmid[c]				
pSJ5[d]	Ap^r	ND[f]	23	W. R. Romig
pSJ8[e]	Cm^r	ND	23	W. R. Romig
pVH1	Ap^r Tc^r Tp^r Sm^r Su^r	C	20, 44	J. Kaper
pVH2	Ap^r Tc^r Km^r	C	20, 44	J. Kaper
Sa	Cm^r Km^r Sm^r Su^r	W	22	N. Datta
Rp4	Ap^r Tc^r Km^r	P	11	N. Datta
Rts1	Km^r	T	10	W. R. Romig
pBR328	Ap^r Tc^r Cm^r		8	M. A. McIntosh
pRK290	Tc^r	P	14	D. Helinski
pRK2013	Km^r	P	14	D. Helinski

[a] Phenotype abbreviations: Ap, ampicillin; Cm, chloramphenicol; Km, kanamycin; r, resistant; Rif, rifampin; Sm, streptomycin; Spc, spectinomycin; Su, Sulfa; Tp, trimethoprim; Vir, virulent.
[b] Spontaneous drug-resistant but fully virulent mutants obtained by plating CA401 on plates containing rifampin (80 μg/ml) or spectinomycin (60 μg/ml).
[c] All plasmids, with the exception of pBR328, are conjugative large plasmids.
[d] *V. cholerae* sex factor marked with Tn1 transposon (P::Tn*1*).
[e] *V. cholerae* sex factor marked with Tn9 transposon (P::Tn9).
[f] ND, Not determined.

Infant mice, 2 to 3 days old and each weighing 2.5 ± 0.05 g, were orally challenged with graded doses of *V. cholerae* cells via a 22-gauge feeding needle (Popper and Son, New Hyde Park, N.Y.). Mice were held for 16 h at 25°C and sacrificed by cervical dislocation. The entire gut (i.e., the intestine plus the stomach) of each mouse was excised and weighed on a Mettler PC400 Delta Range balance (Mettler, Highstown, N.J.). The weight of the remaining carcass (i.e., the body minus the gut) was also recorded. The FA ratio was determined from the following formula: FA ratio = weight of gut/weight of carcass. FA ratios for individual animals were averaged, and the standard error of the mean was calculated. Values reflect the average of six mice per group. An FA ratio of 0.057 ± 0.003 was consistently determined in control group mice, which were inoculated with BHI broth and dye only. FA ratios above 0.066 were considered positive.

In vitro toxin production. Toxin production was assayed by using mouse Y1 adrenal cells in microtiter plates as described previously (36). Y1 cells were maintained on F10 medium supplemented with 12.5% horse serum and 2.5% fetal calf serum (Kansas City Biologicals, Lenexa, Kans.). Toxin production was determined as the maximum dilution of culture supernatant which elicited a positive response on Y1 adrenal cells. *V. cholerae* was cultured in syncase broth (19) at 30°C for 24 h with aeration for CT determinations, and viable counts were taken to adjust the culture density for comparison of CT production. Pure CT (a gift of R. A. Finkelstein, University of Missouri-Columbia, Columbia, Mo.) was used as a control.

Culture supernatant obtained from either JBK70 or JBK70(pVH2) cells grown in syncase medium (as described above) was filtered through membrane filters (pore size, 0.4 μm) and concentrated 10-fold by using an Amicon ultrafiltration cell (Amicon Corp., Danvers, Mass.). Infant mice were fed with the concentrated supernatant at a dose of 0.05 ml per mouse.

DNA manipulations. CA401R was screened for the presence of different plasmids by the method described by Kado and Liu (25). *V. cholerae* chromosomal DNA was prepared by the method described by Marmur (30). Restriction enzymes and T4 DNA ligase were purchased from Bethesda Research Laboratories, Gaithersburg, Md., and used as specified by the manufacturer.

DNA hybridization. The CT gene probe used in the hybridization experiments was the 5.1-kilobase *Pst*I-*Eco*RI restriction fragment from plasmid pJM17 (31). This fragment contains the entire gene for CT (31). The fragment was purified from a low-melting-point agarose gel, and the hybridization was done as described by Maniatis et al. (29). Hybridization was carried out at 50°C. After hybridization, the filter was washed with 3× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 56°C for 2 h.

Stability of plasmids in CA401R. To examine the in vitro stability of all plasmids, CA401R containing different plasmids was grown for 16 h at 37°C in BHI broth and plated on MEA plates to obtain isolated colonies. Two hundred individual colonies were picked onto MEA master plates and replica plated onto MEA plates containing appropriate antibiotics to select for plasmid markers. To examine the in vivo stability of different plasmids, 200 individual colonies from MEA plates (onto which the diluted gut homogenates of infected mice were plated) were picked to MEA master plates and replica plated onto MEA plates containing appropriate antibiotics.

Dual infection competition experiments. Dual infection competition experiments were done as described previously (34). Briefly, strain CA401R containing a plasmid and strain CA401S without plasmids were grown for 18 to 20 h on BHI slants. The inoculum consisted of cells suspended in BHI broth containing 0.01% Evans blue dye and mixed in a 1:1 ratio. Infant mice were challenged orally with a total dose of 1×10^5 to 5×10^5 CFU of *V. cholerae* per mouse in groups of three. The mixed inoculum was plated on MEA plates containing either rifampin or spectinomycin to determine the viable count of each strain. Input ratio was the ratio of CA401R to CA401S in the inoculum.

After 16 h, groups of mice were sacrificed. The upper halves of the small bowels were excised, pooled, suspended in 1.5 ml of BHI, and homogenized in a TRI-R STIR homogenizer (TRI-R Instruments, Rockville Center, N.Y.), and serial dilutions were plated on MEA containing rifampin or spectinomycin. The output ratio was the ratio of CA401R to CA401S colonies in the homogenate.

Statistical analysis. The statistical significance of the results was determined by using the Student *t* test for the estimation of population variance (48).

Growth curves of CA401 and CA401(Rtsl). Strains CA401R and CA401R(Rtsl) were grown in 25 ml of BHI broth in a 125-ml Erlenmeyer flask incubated in a shaking water bath at either 33 or 37°C. Viable counts were made at hourly intervals.

RESULTS

Isolation of rifampin-resistant and spectinomycin-resistant mutants of CA401. For dual competition experiments, we needed strains with different drug resistance markers to allow us to compare the relative abundance of each strain. Drug resistance is also useful property for strain construction. Therefore, we isolated a series of spontaneous rifampin-resistant and spectinomycin-resistant mutants and evaluated their virulence. Most of the rifampin-resistant and spectinomycin-resistant mutants were unable to induce a normal diarrheal response in infant mice, in comparison with the response produced by parental CA401 (Fig. 1). However, one rifampin-resistant mutant, CA401R, and one spectinomycin-resistant mutant, CA401S, which produced near-normal FA responses in mice, were selected for use in this study.

Transmissibility and stability of plasmids. Different R plasmids contained in CA401R (Fig. 2) were transmissible to both *E. coli* HB101 and *V. cholerae* CA401S. Appropriate antibiotic resistance properties were always detected in the recipient strains following conjugal transfer. However, trimethoprim resistance and streptomycin resistance were not detected in plasmid pVH2 after its original transfer to CA401R. No segregation of large R plasmids from CA401R was observed under either in vivo or in vitro conditions. Small plasmid pBR328 readily segregated from both *V. cholerae* and *E. coli* cells in the absence of selection. For CA401R, 23% of colonies recovered from mice and 30% of colonies recovered from 16-h broth cultures had lost the plasmid; 12% of the colonies recovered from 16-h broth cultures of *E. coli* HB101 were cured.

Plasmid effects on the FA response. Plasmids pRK290, Rp4, pBR328, and Sa did not cause a noticeable change in the diarrheal response induced by CA401R ($P > 0.05$) (Fig. 3), nor did plasmids pSJ5 and pSJ8 (*V. cholerae* sex factors with Tn*1* or Tn*9* insertions) alter the FA response produced by CA401R containing them ($P > 0.05$). The response produced by CA401R(pVH2) was significantly higher than the response produced by the control CA401R, while the response produced by CA401R(Rtsl) or CA401R(pVH1) was lower ($P < 0.05$).

Plasmid effects on in vitro toxin production by CA401. Table 2 shows the effects of the plasmids on the ability of CA401R to produce toxin in vitro. Two of the strains [CA401R(pVH2) and CA401R(pVH1)] showed at least a threefold enhancement of CT production, with pVH2 inducing a 12-fold increase. CA401R(Rtsl) produced less than half the toxin produced by control CA401R.

FA response produced by JBK70(pVH2) and its culture supernatant. One of the possible mechanisms by which pVH2 could enhance the virulence of *V. cholerae* was through carrying toxin gene(s). To investigate this possibility, pVH2 was transferred from its CA401S host to a rifampin-resistant mutant of *V. cholerae* JBK70, which is a toxin deletion mutant of strain N16961 (26). Both live cells of JBK70(pVH2) and 10-times-concentrated culture supernatants of JBK70(pVH2) and JBK70 were examined for the ability to induce FA responses in infant mice at 8 and 15 h postinoculation. Concentrated culture supernatant of JBK70(pVH2) produced a detectable FA response, while concentrated supernatant of JBK70 did not (Fig. 4). However, JBK70 cells were able to produce a detectable response if 10^{10} CFU was administered.

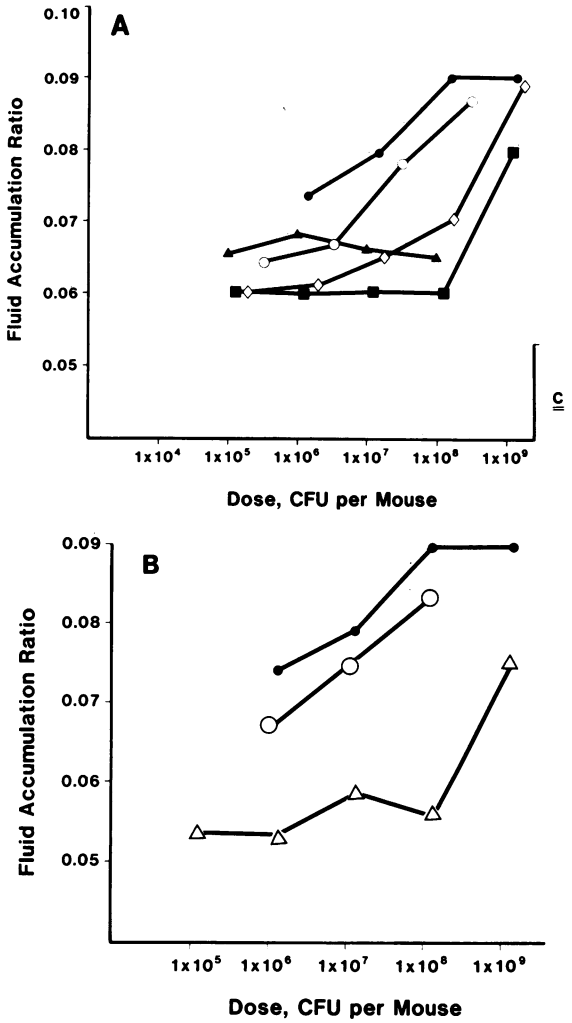

FIG. 1. Dose-related FA ratios of rifampin-resistant and spectinomycin-resistant mutants of *V. cholerae* CA401. Infant mice were orally challenged with various doses of the tested bacteria, and the FA ratio was determined 16 h after challenge. (A) Rifampin-resistant mutants. Symbols: ○, CA401R; ▲, CA401 Rif 101; ◇, CA401 Rif 102; ■, CA401 Rif 103; ●, CA401; C, control (BHI broth only). (B) Spectinomycin-resistant mutants. Symbols: ○, CA401S; △, CA401 spc 100; ●, CA401.

Analysis of pVH2 for CT homologous sequences. The presence of a CT-related DNA sequence in pVH2 was examined by DNA-DNA hybridization experiments; a 5.1-kilobase *Pst*I-*Eco*RI DNA fragment containing the CT gene was used as a probe (31). Under the low-stringency conditions used in

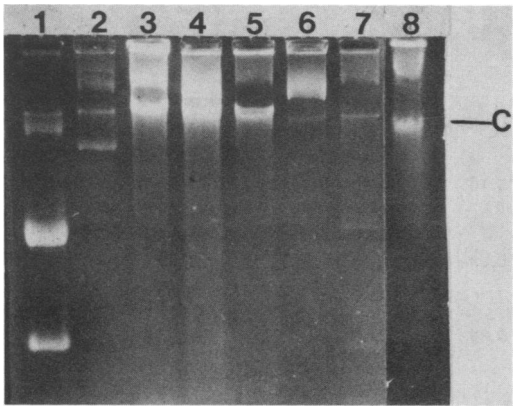

FIG. 2. Agarose gel electrophoresis of plasmid DNA from *V. cholerae* CA401R containing different plasmids. Lanes: 1, pBR328; 2, pRK290; 3, pVH2; 4, pVH2-1; 5, Sa; 6, Rp4; 7, pVH1; 8, pSJ5. C, Chromosomal DNA. The two small plasmids seen in lane 7 are irregularly evident when *V. cholerae* CA401R DNA is prepared by the method of Kado and Liu (25). Such small cryptic plasmids have been found in most *V. cholerae* strains examined (12).

the experiment (3× SSC, 56°C), no DNA sequences homologous to the CT gene were detected (Fig. 5).

Presence of hemolysin genes on pVH2. We looked for hemolysin genes on pVH2 by conjugating the plasmid to *V. cholerae* FA9, a nonhemolytic mutant of CA401 (5). No zone of hemolysis was detected around FA9(pVH2) colonies on sheep blood agar plates.

Effect of pVH2-1 on the response produced by CA401R. Complete digestion of pVH2 with *Pst*I produced two large fragments and four smaller ones. A plasmid derivative (pVH2-1) containing about 80% of the pVH2 DNA was produced by *Pst*I partial digestion and religation of pVH2. This plasmid, tetracycline resistant, kanamycin resistant, and transfer proficient, contained the two large *Pst*I restriction fragments of pVH2. CA401R, to which pVH2-1 was transferred by conjugation, was examined for its ability to induce FA responses in mice. CA401R(pVH2-1) produced a response which was lower than that produced by CA401(pVH2), but still significantly higher ($P < 0.05$) than the response produced by CA401R alone (Fig. 6).

TABLE 2. In vitro CT production[a]

Strain (plasmid)	Amt of CT[b] (ng/ml) ± SD
CA401R	17 ± 2
CA401R(pSJ5)	11 ± 3
CA401R(pVH1)	63 ± 2
CA401R(pVH2)	188 ± 8
CA401R(Sa)	30 ± 12
CA401R(pRK290)	ND[c]
CA401R(RP4)	12 ± 7
CA401R(Rts1)	6 ± 2
CA401R(pBR328)	ND

[a] As determined by adrenal Y1 cell assay with pure CT as a standard.
[b] Average of three experiments.
[c] ND, Not determined.

Plasmid effects on the colonization ability of CA401R. We defined colonization as the ability of a small dose of *V. cholerae* (a dose unable to produce an FA response) to persist and multiply in the upper half of the small intestines of infant mice for 16 to 18 h following challenge (3). In dual

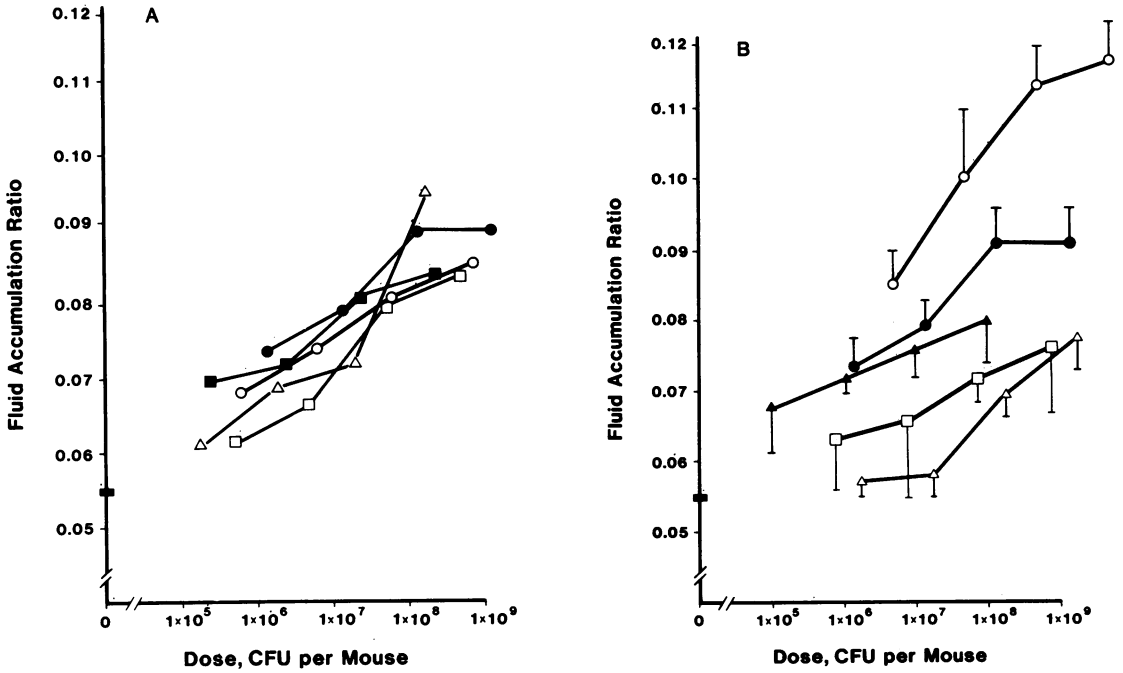

FIG. 3. Dose-related FA ratios of CA401 and CA401R with plasmids. Infant mice were orally challenged with various doses of strain CA401R and its plasmid-containing derivatives, and the FA ratio was determined 16 h after challenge. The FA ratios are shown as the average for 6 to 10 mice. Standard errors of the mean (SEMs) are not shown in panel A for the sake of clarity, but were similar to those in panel B. (A) ●, CA401; ■, CA401R(Rp4); □, CA401R(Sa); ○, CA401R(pRK290); △, CA401R(pBR328); ▬, control (BHI broth only). (B) ●, CA401; ○, CA401R(pVH2); ▲, CA401R(pSJ5); △, CA401R(Rts1); □, CA401R(pVH1). Thin lines in B represent FA ratio ± 1 SEM.

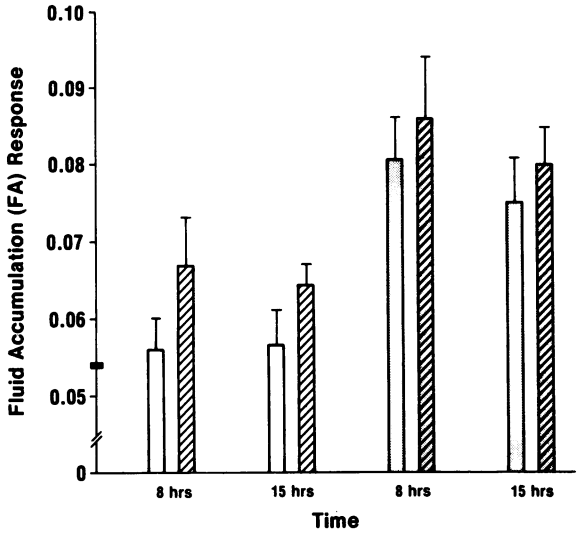

FIG. 4. FA response induced by *V. cholerae* JBK70 and JBK70(pVH2) or 10-fold-concentrated culture supernatant. The dose of JBK70 and JBK70(pVH2) was approximately 10^{10} CFU per mouse. The dose of the 10-fold-concentrated culture supernatant was 0.05 ml per mouse. Open bar, JBK70 culture supernatant; cross-hatched bar, JBK70(pVH2) culture supernatant; shaded bar, JBK70 cells; shaded, cross-hatched bar, JBK70(pVH2) cells. Thin lines represent 1 SEM. Control (10-fold-concentrated syncase broth), ■.

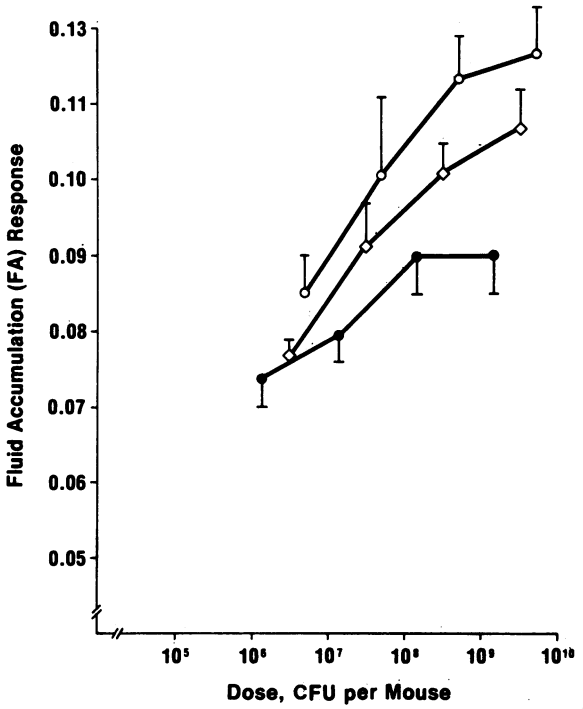

FIG. 6. Dose-related FA ratio of CA401R, CA401R(pVH2), and CA401R(pVH2-1), a deletion derivative of pVH2. Infant mice were orally challenged, and the FA ratios were determined as described in the legend to Fig. 1. Symbols: ●, CA401R; ○, CA401R(pVH2); ◇, CA401R(pVH2-1). Thin lines represent 1 SEM.

infection experiments, 1×10^5 to 2×10^5 CFU of strain CA401R with or without plasmids was compared with an equivalent dose of CA401S given in the same inoculum. *V. cholerae* CA401 was used as a positive control, while *V. cholerae* 596B and VB-12, which colonize the mouse gut poorly (3), were used as negative controls. In comparison with control strains, CA401R containing different R plasmids showed slight reductions in colonization ability (Table 3), but the differences were not considered significant, except for CA401R(pSJ5), which showed a 10-fold decrease, and possibly pVH2, which showed an 8-fold decrease. In replicate experiments with the same strains, only two- to three-

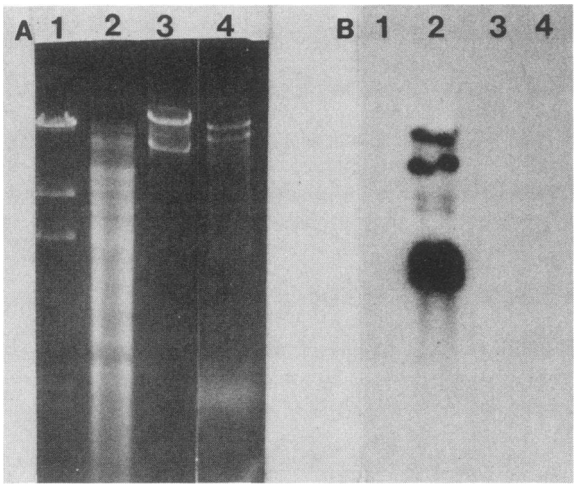

FIG. 5. Hybridization of pVH2 DNA with CT gene probe. (A) Agarose gel containing the following: lane 1, bacteriophage lambda DNA digested with *Hin*dIII for molecular size standards; lane 2, *V. cholerae* chromosomal DNA digested with *Pst*I and *Eco*RI; lane 3, pVH2 digested with *Sal*I; lane 4, pVH2 digested with *Pst*I. (B) Autoradiograph of Southern blot hybridization of the agarose gel in panel A, with a ^{32}P-labeled CT probe.

TABLE 3. Competition experiments to evaluate the effects of plasmids on the ability of CA401 to colonize the infant mouse upper intestine[a]

Type of expt and strain	Input ratio	Output ratio	Change in ratio compared with CA401S (fold decrease)
Controls			
CA401R/CA401	1.5	1.0	None
CA401R/CA401S	1.3	1.0	None
569B/CA401S	3	4×10^{-2}	75
VB12/CA401S	1	1.5×10^{-3}	600
Competition expts			
CA401R(pSJ5)/CA401S	1.6	0.15	10
CA401R(pVH1)/CA401S	1.0	0.25	4
CA401R(pVH2)/CA401S	5.0	0.6	8
CA401R(Sa)/CA401S	2.0	1.0	2
CA401R(RP4)/CA401S	1.0	1.1	None
CA401R(pRK290)/CA401S	5.0	2.0	2
CA401R(Rts1)/CA401S	3.0	0.7	4

[a] *V. cholerae* strains to be tested were mixed to give 1×10^5 to 5×10^5 total CFU/0.05-ml dose. The cell mixture was used to infect three infant mice orally and was diluted and plated on antibiotic plates to determine the input ratio. The mice were sacrificed after 16 h; the upper halves of the small intestines of each group were then removed separately, pooled, homogenized, diluted, and plated on antibiotic plates to determine the output ratio. Single typical experiments are shown, although replicate experiments gave similar results.

fold variations have been observed between dual infection experiments.

In vitro growth curves for CA401R and CA401R(Rts1). CA401R and CA401R(Rts1) were grown to exponential phase in BHI broth at 33°C and inoculated to flasks of BHI broth, which were incubated at either 37 or 33°C with shaking. At each temperature, the growth curves for the two strains were superimposable.

DISCUSSION

The most widely studied virulence factor of *V. cholerae* is CT, which induces massive fluid secretion by intestinal epithelial cells, resulting in diarrhea (18). The role of colonization in the virulence of *V. cholerae* is not clearly defined, although its importance has been suggested by many investigators (1, 21, 33). Infant mouse infection is a sensitive technique for detecting either diarrhea (2) or colonization (3, 4). *V. cholerae* virulence for mice correlates with its virulence for man (38). We have measured both its ability to induce diarrhea and its ability to colonize. These properties are not completely independent of each other; but do reflect different bacterial properties. Positive FA responses with small inocula probably test as much for colonization ability as for toxin production. However, with large inocula, toxin production can be detected even for *V. cholerae* which colonize poorly. The competition tests to measure relative colonization involved the use of a small inoculum, ca. 5×10^5 CFU, which was insufficient to induce a diarrheal response, so that fluid secretion did not interfere with the assay.

Most of the rifampin-resistant and spectinomycin-resistant mutants of the *V. cholerae* strain, CA401, isolated for use in this study caused only low FA responses in mice (Fig. 1). Colonization ability has been shown to be reduced in a rifampin-resistant mutant of *E. coli* (27). However, the rifampin-resistant mutant of CA401 used in this study showed no alteration in its ability to induce diarrhea or in colonization ability, in comparison with its parent strain. Previous reports indicated a role for rifampin resistance in the alteration of bacterial virulence (46). It is likely that spontaneous chromosomal mutations which affect the protein synthesis by pathogenic microorganisms would, in general, affect the virulence of those pathogens.

The FA responses induced by CA401R containing Rp4, pRK290, or pBR328 were equivalent to those induced by the control strain CA401 (Fig. 3). Colonization was also unaffected by the presence of Rp4 or pRK290. Thus, these cloning vectors may properly be used to examine the effect of cloned genes on the in vivo virulence of *V. cholerae*. Because of its rapid segregation, we did not test pBR328 for effects on colonization.

The Tn*1* and Tn*9* derivatives of sex factor P did not affect the FA response or the in vitro toxin production ability of CA401. Colonization ability was depressed slightly by pSJ5. *V. cholerae* sex factors P and V were reported to cause reduction in the virulence of *V. cholerae*, as measured by fluid production in the adult rabbit ileal loop. Sinha and Srivastiva (39) attributed this effect to decreased toxin production. Our results suggest that alterations in other virulence attributes are more likely.

The effects of pVH1 and pVH2 plasmids on FA response and the in vitro toxin production ability was different. CA401R(pVH1) induced a reduced FA response, while CA401R(pVH2) induced a significantly enhanced fluid response, as well as increased in vitro toxin production ability (more than 10-fold increase) (Fig. 3; Table 2). However, CA401R(pVH1), which showed a decreased FA response, had increased in vitro toxin production ability. The ability of *V. cholerae* to induce diarrhea does not always correlate with its in vitro toxin production ability. Levine et al. (28), on the basis of a comparative study between two *V. cholerae* strains for their diarrheagenic ability in human volunteers and their in vitro toxin production ability, concluded that in vitro toxin production by toxigenic strains of *V. cholerae* cannot be a prediction of its virulence for man.

In an attempt to confirm and further examine the virulence-associated nature of pVH2, we transferred it to a *V. cholerae* host from which the CT gene had been deleted. The presence of the plasmid in strain JBK70 caused it to produce a detectable FA response in infant mice, which was seen at 8 and 15 h postinoculation (Fig. 4). However, JBK70, without the plasmid, at the very high dose of 10^{10} CFU per mouse was able to produce a fluid response. A plasmid-mediated enhancement of the fluid response might have occurred. If so, this effect of the plasmid on JBK70 culture supernatant indicates that the plasmid may code for an extracellular virulence-associated factor.

The mechanism by which pVH2 affects the virulence of CA401 is not related to CT itself, as no DNA sequences homologous to the CT gene were detected on the plasmid (Fig. 5). Previous studies have shown that certain strains of *V. cholerae* were able to produce FA responses in mice in the absence of a detectable CT gene (33). This finding led to the suggestion that virulence-related genes, other than those encoding CT, are present in these strains. The absence of any hemolytic activity by *V. cholerae* FA9(pVH2) indicated that no hemolysin gene is present in the plasmid, but it is possible that pVH2 carries virulence-related genes or contains a regulatory gene(s) which controls the expression of chromosomally encoded virulence factors.

Further genetic manipulations of pVH2 included cloning of some of its restriction fragments in plasmid pBR328 and partial deletion analysis. Plasmid pVH2-1, which contains the two large *Pst*I fragments of pVH2 (Fig. 6), was still able to produce an enhancing effect on the FA response of CA401R, suggesting that part or all of the virulence-associated gene(s) encoded by pVH2 is carried on the large *Pst*I fragments.

Plasmid Rts1, a temperature-sensitive plasmid that was maintained stably in *V. cholerae*, was used as a control for other R plasmids used in this study. However, CA401R (Rts1) showed a significant reduction in its ability to produce the FA response and a slight decrease in its in vitro toxin production ability. One explanation for this effect is that Rts1 caused a reduction in the growth rate of cells containing it, even at 33°C, which is the gut temperature of infant mice. However, the in vitro multiplication rate of CA401R(Rts1) was not different from that of CA401R at either 37 or 33°C. Terawaki et al. (43) showed that at temperatures as low as 15°C there was no difference in the multiplication rate of *E. coli* cells containing this plasmid and those lacking it. At 42°C, the multiplication rate of *E. coli* cells containing Rts1 was reduced. Terawaki et al. (42) suggested that the temperature-sensitive replicon of the plasmid might affect the replication of its bacterial host.

In contrast to the control strain, there was a trend for R plasmids to slightly decrease the colonization ability of CA401R containing them (Table 3). The FA responses induced by CA401R containing different plasmids provided one sort of data; the ability to induce a diarrheal response, while dual infection competition experiments provided another. Indirect effects of the plasmids on CA401R could

cause a reduction in colonization ability. Since pVH2 shared this slight decrease in colonization with other plasmids, its virulence-enhancing effect cannot be due to plasmid-encoded colonization antigens, as has been shown for some virulence-related plasmids (16).

In summary, we conclude that plasmids RP4, pRK290, and pBR328, which had no effect on virulence, are suitable vectors for studying the effects of cloned genes on the in vivo virulence of *V. cholerae*. The *V. cholerae* R plasmid pVH2 had a unique enhancing effect on the virulence. This effect was not related to CT, hemolysin, or colonization factors. All R plasmids examined caused a slight reduction of the colonization ability of CA401R; and pVH1 and pVH2 caused elevation of in vitro toxin production.

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