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Role of the P Plasmid in Attenuation of *Vibrio cholerae* O1

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The conjugative plasmid P of *Vibrio cholerae* has been shown to have a suppressive effect on the virulence of hypertoxigenic strains like 569B. In this study, we have sought to analyze this phenomenon. Utilizing the infant mouse cholera model, we have demonstrated that the presence of P increases the 50% lethal dose of *V. cholerae* classical Inaba 569B by more than 300-fold. No effect of P on cholera toxin (CT) production, whether measured by GM_1 enzyme-linked immunosorbent assay, by CT activity in ligated rabbit ileal loops, or by transcription from the CT promoter, could be discerned. Colonization of the intestine by P^+ derivatives was dramatically reduced although only a minor effect could also be demonstrated on in vitro attachment to intestinal strips. Electron microscopic examination suggested that the P plasmid was affecting the production of the TCP pilus. Another conjugative plasmid, V, has also been examined, but it had no effect on virulence.

Within the enteric bacteria, a number of species have been shown to have virulence-related plasmids. Several *Salmonella* spp. require a virulence plasmid ranging in size from about 40 to 100 kilobases (kb) (11, 14, 17). Pathogenic *Escherichia coli*, including enterotoxigenic *E. coli*, enteropathogenic *E. coli*, and enteroinvasive *E. coli* (20), all possess plasmids which encode toxins and factors associated with adherence and invasion. Invasion is also a property associated with the large plasmids found in all virulent *Shigella* species (19, 28, 29). In contrast, plasmids are rare in *Vibrio cholerae* (2).

The induction of disease by *V. cholerae* occurs by the coordinate action of a number of virulence determinants; however, none of these is plasmid encoded. The final diarrheal response is a consequence of a series of events; motility and chemotaxis are employed to penetrate the mucus gel; proteases, DNases, and neuraminidase degrade this gel, and hemagglutinins, including pili, facilitate adherence to the intestinal epithelial cells to allow colonization of the bacteria and permit efficient delivery of cholera toxin (CT) and possibly other toxins.

P is a 68-kb conjugative plasmid capable of self-transfer at high frequency (3, 5, 24). Its main function remains cryptic, but it has been used as a sex factor to mobilize chromosomal markers for genetic mapping of the *V. cholerae* chromosome (26). The V factor is another transmissible plasmid originally identified in a noncholera vibrio (NCV165) (6).

Sinha and Srivastava (34) and Khan et al. (18) observed a 10-fold decrease in the amount of CT produced by classical Inaba strain KB365 (a *str* derivative of 569B) in the presence of the P and V plasmids which they proposed to encode regulatory functions affecting CT biosynthesis. In contrast, Hamood et al. (13) reported that the presence of P neither affected fluid accumulation in infant mice nor diminished in vitro toxin production, as measured by cytopathy of mouse Y1 adrenal cells. It was concluded that if the P factor were attenuating, its influence lay in alterations to virulence attributes other than toxin production.

To clarify the role of plasmids in attenuation, we have investigated the capacity of P and V to affect critical virulence functions of *V. cholerae*, viz., motility, colonization, and toxin production.

MATERIALS AND METHODS

Bacterial strains and cultivation. *V. cholerae* 569B (classical Inaba) and its P^+, V^+, and P^+ V^+ derivatives, as well as KB9 (classical Ogawa) and its V^+ derivative, were from laboratory stocks and were provided by B. S. Srivastava (34). CA401 (classical Inaba) was obtained from C. D. Parker, and V697 is a spontaneous *rif* derivative of CA401 isolated in this study.

Escherichia coli K-12 strains S17-1 (RP4 2-Tc::Mu-Km::Tn*7* *pro hsdR*) and LE392 (F^- *supF supE hsdR galK trpR metB lacY*) were provided by A. Pühler (33) and L. Enquist, respectively.

The plasmids used were pJM17 (21), provided by J. Mekalanos, and pSUP301 and pSUP401 (33), provided by A. Pühler. Plasmid pRU885 (pME305::Tn*1736Tc*) was obtained from R. Schmitt (39).

Nutrient broth (NB) was used as the standard medium for *E. coli* derivatives; it consisted of peptone (Oxoid Ltd.) (10 g $liter^{-1}$), Lab-Lemco (Oxoid) (5 g $liter^{-1}$), and NaCl (5 g $liter^{-1}$). Brain heart infusion broth (BHI, Difco Laboratories) was the standard medium for *V. cholerae* strains. Cultures were incubated with shaking at 37°C. The following antibiotics and concentrations were used: ampicillin, 50 μg/ml; kanamycin, 50 μg/ml; chloramphenicol (CM), 25 μg/ml; tetracycline, 10 μg/ml for *E. coli* and 4 μg/ml for *V. cholerae*.

Strain characterization. The classical Inaba strain 569B was used as the parent strain, and P^+, V^+, and P^+ V^+ derivatives were checked by conjugation and lacuna production (37). The presence of the plasmids was confirmed by the rapid small-scale sodium dodecyl sulfate lysis method (22). As motility is important for virulence (1), all strains were checked for motility by swarming in soft agar as well as for their chemotactic responses to glucose and methionine by a capillary test (10). All strains were verified as equally motile by these two tests.

Construction of pPM854. The 5.1-kb *Pst*I-*Eco*RI fragment of pJM17 harboring the *ctxAB* operon was subcloned into the

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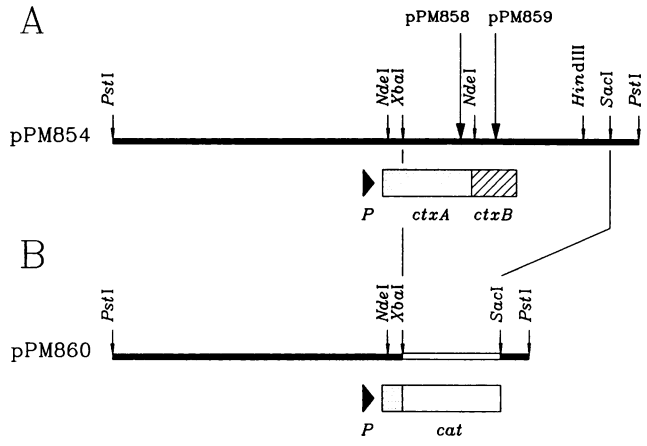

FIG. 1. Construction of plasmids with the CAT gene under the control of the *ctx* promoter. (A) The 5.1-kb fragment encoding the *ctxAB* operon including its promoter (arrowhead) (21) was subcloned into the mobilizable vector pSUP401 (33) to give plasmid pPM854. Plasmids pPM858 and pPM859 are derivatives of pPM854, with the sites of insertion of the transposable promoter probe Tn*1736Tc* (39) in the *ctx* region such that the CAT gene is under the control of the *ctx* promoter. (B) Plasmid pPM860 was constructed by deleting the *Xba*I-*Sac*I segment of pPM854 and replacing it with the promoterless CAT cartridge (open region), in the orientation such that it is under the control of the *ctx* promoter.

mobilizable vector pSUP301 (33). The *Eco*RI site of pJM17 was cleaved and end filled with Klenow fragment of DNA polymerase I, and *Pst*I linkers were added to enable the entire fragment to be subcloned, using *Pst*I into pSUP301, resulting in the inactivation of its β-lactamase (Ap^r) gene. Transformants were screened for Km^r and Ap^s, and plasmid DNA was extracted and examined for the presence of the *ctxAB* fragment. One such isolate was designated pPM854 (Fig. 1).

Promoter detection using Tn*1736Tc*. Insertion of the promoter-detecting transposon, Tn*1736Tc* harboring a promoterless chloramphenicol acetyltransferase (CAT) cartridge, downstream of the *ctx* promoter in plasmid pPM854 was done essentially as described by Ubben and Schmitt (38). The temperature-sensitive plasmid pRU885 (pME305:: Tn*1736Tc*) was introduced into LE392(pPM854) via conjugation, and transconjugants were selected on nutrient agar plus kanamycin plus tetracycline at 30°C. Plasmid DNA was extracted from individual transconjugants cultured at 42°C and transformed into competent LE392 cells, and insertions were selected on plates containing tetracycline and kanamycin. Individual transformants were cultivated for determination of MIC of CM and restriction mapping of the pPM854:: Tn*1736Tc* insertions.

Construction of pPM860. A second approach to measure the effects on the *ctxAB* promoter was the deletion of part of the *ctxA* and *ctxB* genes and the insertion of a promoterless CAT cartridge (Fig. 1). Plasmid pPM1603 is a derivative of pUC19 with the CAT cartridge inserted into the *Bam*HI site (courtesy of A. Barker, this laboratory). Digestion of plasmid pPM854 with *Xba*I and *Sac*I removes a 1.9-kb internal fragment of the *ctxAB* operon. This was replaced with the 0.9-kb *Xba*I-*Sac*I fragment from pPM1603 which includes the CAT cartridge. The resultant ligation was transformed into competent LE392 cells, and transformant colonies were screened for the plasmid with the correct insert. One such isolate, designated pPM860, has the CAT cartridge inserted in the correct orientation for it to be transcribed from the *ctxAB* promoter (Fig. 1).

Inoculation of rabbit ileal loops. Enteropathogenicity in ligated ileal loops of adult rabbits was carried out essentially as described previously (34). Bacterial strains were grown to early exponential phase in BHI broth, and 1-ml samples were injected into the closed ileal loops of adult rabbits. Autopsies were performed after 18 h. The fluid accumulation ratio was the volume (milliliter) of fluid per centimeter of length of ligated loop. Viable counts were performed to determine the inoculum injected into each loop. Before the experiments, rabbits were deprived of food for 24 h and deprived of water for 12 h.

Motility test for *V. cholerae*. Motility was tested by swarming of the bacteria in soft agar and is basically the soft-agar overlay method devised by Stocker (35).

Capillary test for bacterial chemotaxis. The chemotactic ability and motility of a bacterium are directly related. The capillary test for chemotaxis of Freter and O'Brien (10) was employed by using glucose and L-methionine as attractants.

Infant mouse virulence assay. Virulence of strains was determined by using the infant mouse model as described by Attridge and Rowley (1) adapted from Ujiiye et al. (39).

In vitro adherence assay. The adherence of *V. cholerae* to segments of infant mouse small intestine was studied as previously described (1).

In vivo colonization tests. The in vivo colonization capacities of 569B strains with various combinations of the P and V plasmids were compared. Infant mice were fed 5×10^6 bacteria and sacrificed 24 h after challenge; their small intestines were then excised, slit longitudinally, and carefully washed by transfer through three saline baths. The intestines were then homogenized, diluted and plated onto nutrient agar plus streptomycin (100 μg/ml). The recovery of organisms was determined, and the colonies were overlaid with 0.3% soft agar to examine the percentage of motile bacteria.

CT assay. The amount of CT produced in the presence and absence of the P and V factors in *V. cholerae* 569B was measured by the GM_1 ganglioside technique of Holmgren (15). Ganglioside GM_1, the receptor for CT, was obtained from Sigma Chemical Co. Strains were grown in TB plus 66 mM NaCl (pH 6.5) for 18 h at 30°C (23) and the culture supernatant, after filtration through a 22-μm-pore-size Millex filter (Millipore Corp.), was used to measure CT. Anti-CT was raised in rabbits.

Spectrophotometric assay for CAT. Cellular extracts were prepared essentially as described by Close and Rodriguez (8) and spectrophotometrically assayed by the method of Shaw (32). Shaken overnight cultures grown in TB plus 66 mM NaCl at 37°C (23) were diluted 1 in 20 into the same medium and grown to mid-exponential phase at 37°C. Samples (1.5 ml) of cells were harvested and suspended in 0.5 ml of extraction buffer (50 mM Tris hydrochloride [pH 7.8], 30 μM dithiothreitol). The cells were disrupted by using a Branson sonicator, and the extract was cleared of cell debris by centrifugation at 15,000 rpm for 15 min at 4°C in a Sorvall SM24 rotor. The supernatant was assayed for CAT activity in a 1-ml reaction volume. The rate of increase in A_{412} was measured to determine enzyme units in nanomoles of dithiobis-nitrobenzoic acid reduced per minute per milligram of total protein. Protein was assayed by the method of Bradford (7) by using a dye reagent concentrate (Bio-Rad Laboratories) and bovine serum albumin (Sigma) as the standard.

Electron microscopy. Cells grown on CFA (9a) agar for 36 h at 25°C were suspended in phosphate-buffered saline (pH

TABLE 1. Fluid accumulation and CT production of the 569B strain series

Strain and plasmid	Fluid accumulation ratio in rabbit ileal loops (ml/cm ± SD) (n = 4)	CT production[a] (μg/ml)
None (BHI)	0 ± 0	
569B	1.56 ± 0.33	1.2 ± 0.13
569B P^+ V^+	1.49 ± 0.40	1.4 ± 0.10
569B P^+	1.65 ± 0.30	1.5 ± 0.12
569B V^+	1.57 ± 0.33	1.5 ± 0.12

[a] Concentration of CT was determined by the GM_1 ganglioside ELISA (15). The sensitivity of the assay was 0.02 μg of CT per ml.

7.2). A drop (35 μl) of cell suspension was placed on a sheet of parafilm. A poly-L-lysine-treated Formvar-coated electron microscope grid was then placed, plastic side down, on the surface of this drop for 2 min. The grids were then negatively stained with 2% (wt/vol) uranyl acetate and examined in a Philips EM300 microscope at 80 kV.

RESULTS

Production of CT in the presence of P and V plasmids. In an initial attempt to detect differences in CT production in the presence of the P and V plasmids, fluid accumulation in ligated ileal loops of adult rabbits after administration of 569B, 569B P^+, 569B V^+, and 569B P^+ V^+ was investigated. Samples of each of the strains were introduced into ligated ileal loops, and the mean fluid accumulation ratio was calculated from loops constructed in four rabbits (Table 1). No differences in the fluid accumulation ratio were observed.

Use of the GM_1 ganglioside enzyme-linked immunosorbent assay (ELISA) (15) indicated that the amount of CT was also unaffected by the presence of the P or V plasmids (Table 1).

CT promoter analysis. The genes coding for CT biosynthesis, *ctxAB*, have been cloned as a *Pst*I-*Eco*RI fragment in pJM17 (21). This cloned fragment includes the promoter and regulatory region for these genes. To rule out any possible effects by the P and V factors on the transcription at the *ctx* promoter operon, fusions were constructed (Fig. 1).

Plasmids pPM858 and pPM859 containing the transposable promoter probe Tn*1736Tc* (39), which contains a promoterless CAT gene map approximately 570 and 880 base pairs downstream from the *ctx* promoter, are located in *ctxA* and *ctxB*, respectively. The plasmids were mobilized into classical *V. cholerae* 569B and CA401 in the presence and the absence of the P and V factors. The amount of CAT produced in each strain was assayed both spectrophotometrically and by determining the MICs of CM (Table 2).

Plasmids pSUP401 (encoding Cm^r) and pSUP301 were included as controls. The amount of CAT produced by pSUP401 was comparable with that reported previously (4). In agreement with the results found by other investigators, *V. cholerae* produces a lower level of resistance to CM than *E. coli* K-12 does (25). As expected, neither pPM854 (the *ctxAB* operon subcloned into pSUP301) nor pSUP301 exhibited any resistance to CM. Surprisingly, the level of Cm^r in *V. cholerae* CA401 was slightly higher than in 569B.

Plasmids pPM858 and pPM859 contain Tn*1736Tc* inserted in the correct orientation such that CAT is under the control of the *ctxAB* promoter. The level of resistance is significantly lower than for pSUP401, indicating that the *ctxAB* promoter is not as strong as the natural CAT promoter (36). Plasmid pPM860 exhibits the highest level of CAT of this series, which is not surprising since it has the added benefit of the more efficient *ctxB* ribosome-binding site. The CAT activities of none of the plasmids (pPM858, pPM859, or pPM860) were affected by the presence or absence of the P and V factors, suggesting that P is not suppressing virulence by affecting the transcription of the CT genes.

Virulence in the infant mouse model. The effects of P and V on virulence were investigated by determining 50% lethal dose (LD_{50}) values for the 569B strain series in the infant mouse model. The LD_{50} values (geometric means ± standard deviations from two to four separate determinations by the method of Reed and Muench [27]) for the 569B strain series were as follows: (2.0 ± 1.5) × 10^5 for 569B; >10^8 for 569B P^+ V^+; >10^8 for 569B P^+; and (2.1 ± 0.9) × 10^5 for 569B V^+. The virulence of 569B was unaltered by the presence of V, whereas the presence of P led to more than a 500-fold increase in LD_{50}, whether or not V was also present.

Colonization. The attenuating effect of P on the 569B strain prompted us to examine the effect of this plasmid on the motility and colonization of 569B. The motility of *V. cholerae* and its ability to move toward L-methionine and glucose was not affected by P (or V [data not shown]).

To examine the colonization potential of the 569B strain series, four groups of six infant mice were fed 5 × 10^6 organisms, a dose which would not kill the mice within 24 h. At this time, the mice were sacrificed, their small intestines were carefully excised, and bacterial contents were enumerated. In the presence or absence of V, P had a dramatic effect on the ability of 569B to persist in the gut (Table 3). These data indicate that the P plasmid inhibits either the

TABLE 2. Measurement of CAT activity in *CTX* operon fusions

Plasmid	CAT production of *V. cholerae* O1 (V697)[a]				MIC (μg/ml) of CM under the control of *ctxAB promoter* for *V. cholerae* O1							
					569B				CA401			
	P^- V^-	P^+ V^+	P^+	V^+	P^- V^-	P^+ V^+	P^+	V^+	P^- V^-	P^+ V^+	P^+	V^+
No plasmid	3	3	3	3	1	1	1	1	1	1	1	1
pSUP401	160	138	179	130	50	50	50	50	100	100	100	100
pSUP301	3	5	4	4	1	1	1	1	1	1	1	1
pPM854	2	3	3	4	1	1	1	1	1	1	1	1
pPM858	10	10	11	12	25	25	25	25	50	50	50	50
pPM859	8	8	6	9	25	25	25	25	25	25	25	25
pPM860	40	48	48	40	50	50	50	50	100	100	100	100

[a] CAT assay was performed as described by Shaw (32) and calculated as micromoles per minute per milligram of total protein. Total protein was determined by the method of Bradford (7). V697 is a Rif^r derivative of CA401.

TABLE 3. In vivo colonization and in vitro adherence of the 569B strain series

Strain and plasmid	Colonization of small intestine[a] GM ± SE (log_{10})	In vitro attachment (%)[b]		
		NB at 37°C		CFA at 25°C
		Expt 1	Expt 2	
569B	7.77 ± 0.05	4.8	7.7	7.2
569B P^+	2.27 ± 0.67[c]	2.7	7.1	4.0
569B P^+ V^+	2.20 ± 0.89	1.0	6.9	4.8
569B V^+	6.98 ± 0.68	4.3	9.6	10.0

[a] Persistence of bacteria in the small intestine of infant mice 24 h after dosing.

[b] Total adherence (%) is the total number of bacteria recovered from the four intestinal strips divided by the total number of bacteria added.

[c] One mouse in the group of six showed no colonies on a neat plate; one colony was used for statistical purposes.

initial colonization or subsequent in vivo growth of the 569B strain. The latter possibility was investigated by examining growth characteristics in minimal medium (MM) and the extent of a lag phase on transfer from a rich to a poor medium (BHI to MM) and confirming the prototrophic nature of the strains. No effect on any of these properties was detected (data not shown), suggesting that the P plasmid is more likely to reduce the initial colonization of the 569B strain.

In vitro adherence. An in vitro adherence assay was used to examine the effect of the P and V factors on the capacity of *V. cholerae* 569B to bind to infant mouse intestinal tissue. When the strains were grown under routine culture conditions (Table 3, NB at 37°C) the presence of P or V had no effect on the level of attachment.

Work from several laboratories has now strongly implicated the TCP pilus as being important for colonization and virulence (30, 31, 38). The various 569B derivatives were therefore grown under conditions optimal for TCP expression (Table 3, CFA agar at 25°C) to see whether P had any effect on production of these pili. Electron microscopy (Fig. 2) revealed a difference in the amount of TCP produced in the presence of the P plasmid. Whereas thick bundles of TCP filaments were readily detected in plasmid-free 569B or in the presence of V, only a few single filaments were detected in the presence of P. When such suspensions were assayed for in vitro attachment to intestinal strips, those bacteria carrying the P plasmid were slightly less able to bind (Table 3, CFA at 25°C).

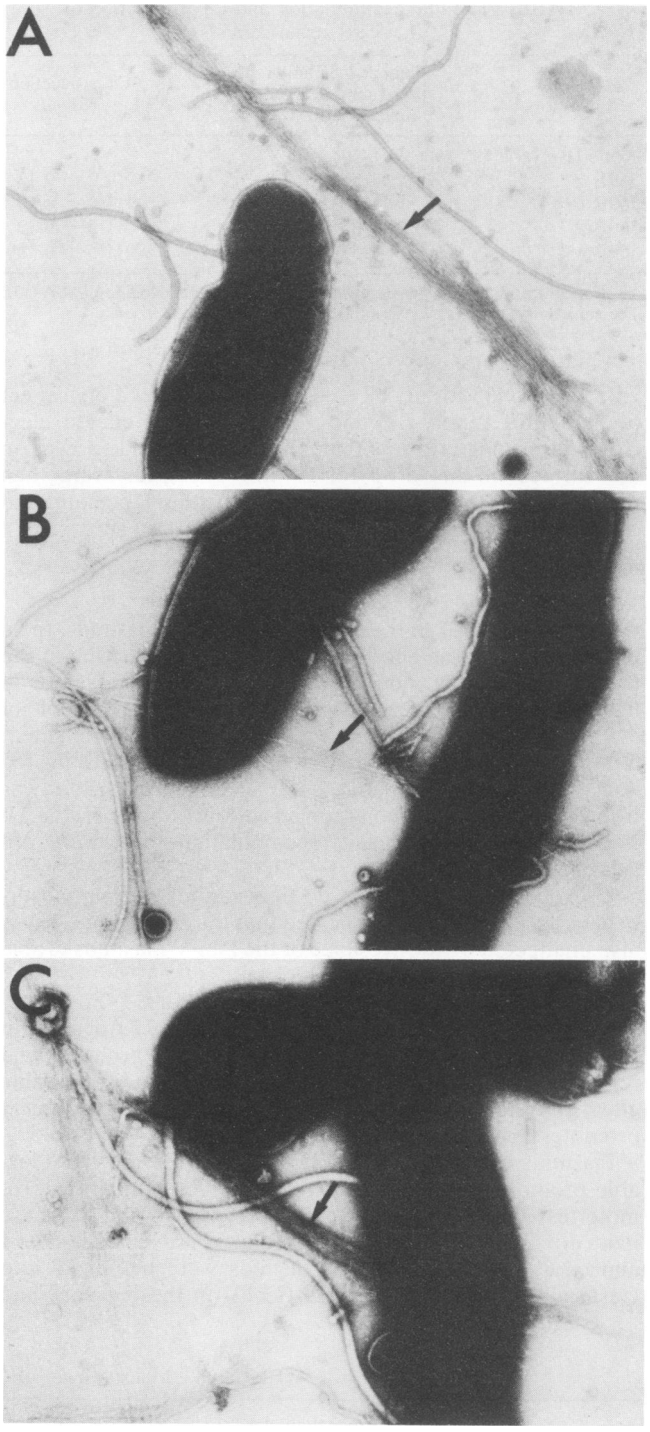

FIG. 2. Electron microscopic photographs of strains. The strains were grown on CFA agar at 25°C for 36 h, conditions previously shown to be optimal for expression of TCP pilus (30, 31). The grids were stained with uranyl acetate. (A) 569B; (B) 569B P^+; (C) 569B V^+. The TCP pili are indicated by arrows.

DISCUSSION

A series of derivatives of *V. cholerae* 569B has been used to evaluate the effects of the P and/or V plasmids on infant mouse virulence. Our data show that P, in the presence or absence of V, is dramatically attenuating for this strain; V alone has no effect. Investigation of the mechanism by which P might exert its effect did not support previous reports (18, 34) that this plasmid may inhibit the biosynthesis of CT. Whether compared in a rabbit ileal loop system or by a GM_1 ELISA on culture supernatants, CT production by P^+ and P^- strains was equivalent. Moreover, transcription from the *ctxAB* promoter was not reduced in strains carrying the P plasmid. The basis for the difference between our findings and those of Srivastava and colleagues is not readily apparent.

It was then of interest to examine other properties of the P^+ strains which may have impact upon pathogenic potential. The vibrio flagellum has been shown to play important roles in attachment in vitro and colonization in vitro (1, 9, 16), although there has been some debate as to whether this structure functions merely as an agent of motility or whether it also carries an adherence factor (1). However, the 569B P^+

strains showed undiminished motility, both in soft agar and in chemotaxis assays. Moreover, the in vitro attachment of P^+ and P^- strains was found to be similar in a system in which the putative flagellar adhesin is thought to play an important role.

As expected from their much higher LD_{50} values, 569B P^+ and 569B P^+ V^+ were recovered in much lower numbers than the 569B and 569B V^+ strains, when intestines were excised from mice fed 24 h earlier. Evidently the P^+ derivatives colonize less effectively initially or multiply less rapidly thereafter or both. In vitro comparisons of the growth rates of the 569B strain series in MM failed to reveal any effect of the P plasmid on generation time. However, the P^+ strains did show a reduced production of TCP when cultured under conditions previously shown (12, 31) to promote synthesis of such pili by *V. cholerae* 569B. In view of the critical role of TCP in the colonization of the infant mouse intestine (30, 31), our findings therefore favor the hypothesis that the avirulence of P^+ strains results from impaired initial colonization. When the four 569B variants were cultured under conditions conducive to TCP expression, those carrying the P plasmid were slightly less adherent in vitro.

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