Suppression of Pathogenicity by P and V Plasmids in *Vibrio cholerae*

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In experimental models of cholera, some strains of *Vibrio cholerae* harbouring P or V or both plasmids were markedly less pathogenic than isogenic strains devoid of these plasmids. This effect was due to decreased production of toxin by P⁺, V⁺ or P⁺V⁺ bacteria compared with their parents.

INTRODUCTION

Some vibrio strains are known to harbour two plasmids designated P and V (Bhaskaran, 1960; Bhaskaran & Sinha, 1971). While the former has been characterized as a sex factor promoting conjugation in *Vibrio cholerae*, the role of the latter is not clear. Both plasmids can coexist in a cell but conjugation promoted by P is repressed in the presence of V (Bhaskaran & Sinha, 1971). There is no report that these plasmids are associated with virulence in *V. cholerae*. In *V. cholerae*, the gene *tox* coding for the synthesis of an enterotoxin protein, has been mapped on the chromosome and is closely linked to the *his* locus (Vasil, Holmes & Finkelstein, 1975). The gene controlling synthesis of enterotoxin in *Escherichia coli* has been shown to be located on a plasmid (Williams Smith & Halls, 1968; Skerman, Formal & Falkow, 1972). Recently, R and ColI plasmids have been found to increase the resistance of *E. coli*, *Salmonella typhimurium* and *Pseudomonas aeruginosa* to radiation and chemical treatments (Howarth, 1965; Marsh & Smith, 1969; Tweats *et al.*, 1976; MacPhee, 1972; Mortelmans & Stocker, 1976; Lehrbach *et al.*, 1977). We report here that the presence of P and/or V plasmids of *V. cholerae* renders the bacteria less pathogenic in experimental models of cholera and that this effect is due to decreased toxin production.

METHODS

Vibrio strains with their relevant markers are listed in Table 1. All strains were maintained on Difco nutrient agar slants in test tubes at 4 °C.

Media. Broth cultures of bacteria were in Difco Bacto Brain Heart Infusion (BHI) at 37 g per l distilled water. Syncase sucrose minimal medium was prepared as described by Finkelstein & LoSpalluto (1969). The composition of the minimal medium supplemented with other nutritional requirements was described by Bhaskaran & Sinha (1971). Phosphate-buffered saline (PBS, pH 7.2), used for bacterial dilution, consisted of K₂HPO₄ (1.21 g l⁻¹), KH₂PO₄ (0.34 g l⁻¹) and NaCl (8.0 g l⁻¹). Granular mucin (type 1701-W, Wilson Laboratories, Chicago, Illinois, U.S.A.) was prepared by dissolving 50 g in 1 l distilled water; after sterilization the pH was adjusted to 7.2 with sterile NaOH.

Broth culture. Overnight slant cultures were suspended in 8 ml PBS. This suspension was diluted 50-fold in BHI and incubated for 3 h without shaking at 37 °C; these cultures had a viable count of about 2 × 10⁹ vibrios ml⁻¹.

Construction of strains with P and/or V factors. The method for isolating and characterizing P⁺, V⁺ and P⁺V⁺ strains of KB9 (earlier designation v58:SR) has been described (Bhaskaran & Sinha, 1971). Using the same techniques, P⁺, V⁺ and P⁺V⁺ derivatives of 569B were isolated by separate crosses with P⁺ and V⁺ strains of KB9, selecting for *ilv*⁺ recombinants (KB9:P × 569B; KB9:V × 569B; KB9:V × 569B:P). Similarly,

Table 1. *Vibrio strains*

Strain	Relevant markers	Plasmid status	Reference or source
<i>Vibrio cholerae</i>			
KB9	<i>ilv arg his</i>	P-V-	Bhaskaran & Sinha (1971)
KB9:P	<i>ilv arg his</i>	P+V-	Bhaskaran & Sinha (1971)
KB9:V	<i>ilv arg his</i>	P-V+	Bhaskaran & Sinha (1971)
KB9:PV	<i>ilv arg his</i>	P+V+	Bhaskaran & Sinha (1971)
569B		P-V-	K. Bhaskaran
569B:P		P+V-	This work
569B:V		P-V+	This work
569B:PV		P+V+	This work
KB92		P-V-	K. Bhaskaran
KB92:P		P+V-	This work
KB378	<i>str</i> , non-motile	P-V-	K. Bhaskaran
KB378:P	non-motile	P+V-	This work
<i>Vibrio el Tor</i>			
KB207	<i>str</i>	P-V-	K. Bhaskaran
KB207:P	<i>str</i>	P+V-	This work
KB381	<i>str</i>	P-V-	K. Bhaskaran
KB381:P	<i>str</i>	P+V-	This work

KB92:P was isolated by mating KB9:P and KB92, and P+ derivatives of KB378, KB207 and KB381 were isolated by mating with KB92:P and selection on media supplemented with streptomycin.

Enteropathogenicity in ileal loops of adult rabbits. Broth cultures (3 h) were diluted in PBS and 1 ml samples were injected into closed ileal loops of adult rabbits. Autopsies were performed after 18 h (De & Chatterje, 1953). Outpouring of fluid was usually observed with a virulent strain. Ileal loops with accumulated fluid were scored as positive and loops without fluid as negative. Albino rabbits, weighing 1 to 1.5 kg, reared in our Institute and starved for 48 h before the experiments, were used.

Virulence in Swiss mice. Broth cultures (3 h) of various vibrio strains were diluted in mucin as follows: KB9, KB92 and their P+, V+ derivatives to 10^{-2} , 5×10^{-7} and 10^{-8} ; and KB378, KB207, KB381 and their P+ derivatives to 10^{-2} , 10^{-3} and 10^{-4} . Swiss mice weighing 14 to 16 g and reared in this Institute were taken in groups of 10; one group was used for each bacterial dilution. Mice were injected intraperitoneally with 0.5 ml of mucinized culture suspensions and death was recorded up to 72 h. Virulence was expressed as LD₅₀, determined by the method of Reed & Muench (1938).

Preparation and assay of Vibrio enterotoxin. Crude toxin was prepared by growing *V. cholerae* in Syncase sucrose medium at 28 °C for 16 h in a shaker. The culture was centrifuged and the supernatant, which contained the crude enterotoxin, was gently removed and stored at 4 °C. Crude enterotoxin was diluted 10-fold in BHI and filtered through a 'Metricel' membrane (Gelman Instrument Co., Ann Arbor, Michigan, U.S.A.; pore size, 0.2 µm). Serial dilutions of the filtrate were made in BHI and 0.1 ml samples were injected intradermally in adult albino guinea-pigs. Induration (dome-shaped firm swelling) was recorded after 18 h.

RESULTS

Enteropathogenicity in adult rabbits

Taking the same size of inoculum, the pathogenicity of P-V-, P+, V+ and P+V+ strains was compared in adult rabbits (Table 2). The tests showed a significant difference in ileal loop distension between the KB9, KB9:P, KB9:V and KB9:PV suspensions. There was a greater outpouring of fluid in loops with KB9 than with its plasmid-carrying derivatives, suggesting that the former was more pathogenic. There was no difference in the pathogenicity of P+ and P- strains of the *el Tor* biotype.

A positive loop of KB9 and a negative loop of KB9:P inoculated with approximately the same number of cells were compared for final viable counts. No difference was observed - the counts were 1.8×10^9 ml⁻¹ and 1.3×10^9 ml⁻¹ respectively. Therefore the difference in pathogenicity may not be due to poor growth of P+ strains. No difference in growth *in vitro* or in the capacity to adhere to the intestine was obtained between P- and P+ strains (unpublished results).

Table 2. Comparison of pathogenicity of *P*⁻*V*⁻, *P*⁺, *V*⁺ and *P*⁺*V*⁺ strains in the ileal loops of adult rabbits

Data are from one experiment out of three which gave very similar results. Results are expressed as the amount of fluid distension (ml per cm loop length).

Dilution of dose*	Vibrio strain					
	KB9 (1.3×10^9 ml ⁻¹)†	KB9:P (1.5×10^9 ml ⁻¹)	KB9:V (2.0×10^9 ml ⁻¹)	KB9:PV (2.1×10^9 ml ⁻¹)	KB381 (1.4×10^9 ml ⁻¹)	KB381:P (1.2×10^9 ml ⁻¹)
10 ⁻⁴	1.73	1.85	0.00	0.00	1.77	1.86
10 ⁻⁵	1.13	0.00	0.00	0.00	1.77	1.85
10 ⁻⁶	0.92	0.00	0.00	0.00	ND	ND

ND, Not done.

* Samples (1 ml) of the dilution shown were injected into the ileal loop of rabbits as described in Methods.

† Titre of initial cultures.

Table 3. LD₅₀ of different *P*⁻*V*⁻, *P*⁺, *V*⁺ strains in mouse virulence tests

Vibrio strain	LD ₅₀ (no. of bacteria)	Vibrio strain	LD ₅₀ (no. of bacteria)
KB9	< 9	KB207	2.4×10^4
KB9:P	69	KB207:P	2.0×10^4
KB9:V	86.7	KB381	2.4×10^6
KB92	< 13	KB381:P	2.7×10^6
KB92:P	59.3		
KB378	2.5×10^5		
KB378:P	7.2×10^6		

Mouse virulence

Mice do not display the typical pathological manifestation of experimental cholera. However, mice have been used as experimental models for quantifying virulence. Various vibrio strains harbouring the plasmids P and V (Table 1) were injected intraperitoneally and the LD₅₀ of each was calculated (Table 3). The LD₅₀ values of *P*⁺ and *V*⁺ strains were higher than those of their isogenic *P*⁻*V*⁻ strains and therefore strains carrying the plasmids exhibited less virulence in this model than their *P*⁻ or *V*⁻ counterparts. There was no difference in the LD₅₀ values of *P*⁺ and *P*⁻ derivatives of *V. el Tor*.

Assay of cholera enterotoxin

The intradermal reaction to cholera toxin in guinea-pigs was demonstrated by Craig (1965) and has been used to assay toxin. Inaba 569B is a highly toxigenic strain *in vitro* and so *P*⁺, *V*⁺ and *P*⁺*V*⁺ derivatives of this strain were isolated and used in this study. Cholera enterotoxin was prepared from these strains (569B, 569B:P, 569B:V and 569B:PV) and was titrated in guinea-pigs. Induration produced by various batches of toxin was recorded (Table 4). A significant difference was observed between 569B and its *P*⁺, *V*⁺ or *P*⁺*V*⁺ derivatives; the plasmid-carrying strains produced less enterotoxin as measured by indurated lesions. Strains 569B:P, 569B:V and 569B:PV were identical in this respect.

DISCUSSION

We have demonstrated that the virulence of *V. cholerae* can be suppressed by the presence of P and/or V plasmids. This effect is attributed to a decreased level of toxin production by plasmid-carrying cells. This is a novel property of a plasmid. P and V plasmids together in the same cell had no additive effect on toxin production. Bhaskaran & Sinha (1971) reported that when the P plasmid was transferred into a cell in which there was a resident V plasmid, the former was repressed. An analogous situation may be occurring in the experiments reported here so that an additive effect of P and V plasmids was not evident.

Table 4. *Assay of cholera toxin in guinea-pigs*

Vibrio strain	Dilution of toxin	Induration (mm) caused by different batches of toxin					
		I	II	III	IV	V	VI
569B	10 ⁻²	20	18	19	17	18	18
569B	10 ⁻³	12	12	14	13	11	14
569B	10 ⁻⁴	0	0	0	0	0	0
569B:P	10 ⁻²	13	0	13	13	16	14
569B:P	10 ⁻³	0	0	0	0	9	0
569B:P	10 ⁻⁴	0	0	0	0	0	0
569B:V	10 ⁻²	14	13	14	15	ND	ND
569B:V	10 ⁻³	0	0	12	0	ND	ND
569B:V	10 ⁻⁴	0	0	0	0	ND	ND
569B:PV	10 ⁻²	14	14	10	11	ND	ND
569B:PV	10 ⁻³	0	0	0	0	ND	ND
569B:PV	10 ⁻⁴	0	0	0	0	ND	ND

ND, Not done.

In other bacteria, plasmids conferring resistance to ultraviolet ionizing radiation and alkylating agents have been shown to code for the enzyme(s) participating in error-prone repair pathways (Mortelmans & Stocker, 1976; Lehrbach *et al.*, 1977). In *V. cholerae*, the P and V plasmid gene product(s) may interfere in the synthesis or release of enterotoxin. In some cases, R plasmids are known to modify the permeability of bacterial cells. For example, plasmid-controlled resistance to tetracycline in *Staphylococcus aureus* and *Escherichia coli* is due to decreased uptake of the drug (Sompolinsky *et al.*, 1970; Franklin, 1967).

We do not know why *V. el Tor* strains are refractory to the effect of the P plasmid. *Vibrio el Tor* differs from *V. cholerae* in that P is repressed unless the bacteria are exposed to a thermal shock at 44.5 °C (Bhaskaran, Sinha & Iyer, 1973) or are resistant to group IV phage (Mukherjee, 1963) or polymyxin (Gan & Tjia, 1963). Therefore the lack of plasmid effect could be due to the plasmid not being expressed.

The practical importance of this investigation is in the preparation of a live vaccine whose safety may be enhanced by inclusion of P and/or V factors. Little work has been done on the curing of vibrio cells of plasmids, though Parker *et al.* (1972) have tested a variety of substances which cure *E. coli* of F, F' and R factors, but without success. From our own observations, it appears that P and V plasmids in *V. cholerae* are not easily lost either *in vitro* or by passage into the rabbit intestine (unpublished results). It remains to be seen whether bacterial antigenicity will also be affected by the plasmids.

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REFERENCES

- BHASKARAN, K. (1960). Recombination of characters between mutant stocks of *Vibrio cholerae*, strain 162. *Journal of General Microbiology* **23**, 47–54.
- BHASKARAN, K. & SINHA, V. B. (1971). Transmissible plasmid factors and fertility inhibition in *Vibrio cholerae*. *Journal of General Microbiology* **69**, 89–97.
- BHASKARAN, K., SINHA, V. B. & IYER, S. S. (1973). Chromosome mobilization in *Vibrio cholerae* (biotype eltor) mediated by sex factor P. *Journal of General Microbiology* **78**, 119–124.
- CRAIG, J. O. (1965). The effect of cholera stool and culture filtrates on the skin of guinea-pigs and rabbits. In *Proceedings of the Cholera Research Symposium* 1965, Honolulu, Hawaii, pp. 153–158. Washington: U.S. Government Printing Office.
- DE, S. N. & CHATTERJE, D. N. (1953). An experimental study of the mechanism of action of *Vibrio cholerae* on the intestinal mucous membrane. *Journal of Pathology and Bacteriology* **66**, 559–562.

- FINKELSTEIN, R. A. & LoSPALLUTO, J. J. (1969). Pathogenesis of experimental cholera: preparation and isolation of cholera toxin and cholera toxinogen. *Journal of Experimental Medicine* **130**, 185–202.
- FRANKLIN, T. J. (1967). Resistance of *Escherichia coli* to tetracyclines. Changes in permeability to tetracyclines in *Escherichia coli* bearing transferable resistance factors. *Biochemical Journal* **105**, 371–378.
- GAN, K. H. & TJIA, S. K. (1963). A new method for differentiation of *Vibrio comma* and *Vibrio el Tor*. *American Journal of Hygiene* **77**, 184–186.
- HOWARTH, S. (1965). Resistance to the bactericidal effect of ultraviolet radiation conferred on Enterobacteria by the colicin factor *ColI*. *Journal of General Microbiology* **40**, 43–55.
- LEHRBACH, P., KUNG, A. H. C., LEE, B. T. O. & JACOBY, G. A. (1977). Plasmid modification of radiation and chemical-mutagen sensitivity in *Pseudomonas aeruginosa*. *Journal of General Microbiology* **98**, 167–176.
- MACPHEE, D. G. (1972). Effect of an R factor on resistance of *Salmonella typhimurium* to radiation and chemical treatment. *Mutation Research* **14**, 450–453.
- MARSH, E. B., Jr & SMITH, D. H. (1969). R factors improving survival of *Escherichia coli* K-12 after UV irradiation. *Journal of Bacteriology* **100**, 128–139.
- MORTELMANS, K. E. & STOCKER, B. A. D. (1976). Ultraviolet light protection, enhancement of ultraviolet light mutagenesis and mutation effect of plasmid R46 in *Salmonella typhimurium*. *Journal of Bacteriology* **128**, 271–282.
- MUKERJEE, S. (1963). The bacteriophage susceptibility tests in differentiating *Vibrio cholerae* and *Vibrio el Tor*. *Bulletin of the World Health Organization* **28**, 333–336.
- PARKER, C., GAUTHIER, D., TATE, A. & ROMIG, W. R. (1972). Recombination in *Vibrio cholerae*. In *Proceedings of the 7th Joint Conference, U.S.–Japan Cooperative Medical Science Programme, Cholera Panel, Symposium on Cholera*, Woods, Massachusetts, August 1971, pp. 24–34.
- REED, L. J. & MUENCH, H. (1938). A simple method for estimating fifty percent endpoints. *American Journal of Hygiene* **27**, 493–497.
- SKERMAN, F. J., FORMAL, S. B. & FALKOW, S. (1972). Plasmid associated enterotoxin production in a strain of *Escherichia coli* isolated from humans. *Infection and Immunity* **5**, 622–624.
- SOMPOLINSKY, D., ZAIDENZAIG, Y., ZIEGLER-SCHLOMOWITZ, R. & ABRAMOVA, N. (1970). Mechanism of tetracycline resistance in *Staphylococcus aureus*. *Journal of General Microbiology* **62**, 351–362.
- TWEATS, D. J., THOMPSON, M. L., PINNEY, R. J. & SMITH, J. T. (1976). R factor-mediated resistance to ultraviolet light in strains of *Escherichia coli* deficient in known repair functions. *Journal of General Microbiology* **93**, 103–110.
- VASIL, M. L., HOLMES, R. K. & FINKELSTEIN, R. A. (1975). Conjugal transfer of a chromosomal gene determining production of enterotoxin in *Vibrio cholerae*. *Science* **187**, 849–850.
- WILLIAMS SMITH, H. & HALLS, S. (1968). The transmissible nature of the genetic factor in *Escherichia coli* that controls enterotoxin production. *Journal of General Microbiology* **52**, 319–334.