Regulation of Toxin Biosynthesis by Plasmids in *Vibrio cholerae*

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Vibrio cholerae strain 569B Inaba harbouring P plasmid produced less toxin than the parent strain. To examine the effect of plasmid loss on toxin production, temperature-sensitive (ts) mutants of P, unable to replicate at 42 °C, were isolated. One ts plasmid was unstable at 42 °C and its loss yielded a cured strain that resumed a normal level of toxin biosynthesis characteristic of the plasmid-free parent strain. Toxin production was again suppressed in the cured strain after reacquisition of P plasmid. This suggested a role for plasmid-borne genes in the regulation of toxin biosynthesis. A mutant of strain 569B Inaba that produced mutant toxin was isolated by transfer of P and V plasmids. The mutant toxin was similar to cholera toxin because it did not give rise to symptoms of cholera but induced antitoxin immunity in rabbits.

INTRODUCTION

Cholera toxin produced by *Vibrio cholerae* is a multimeric protein consisting of one A and five B subunits (Gill, 1976), and the genes encoding and regulating toxin biosynthesis appear to be located on the *V. cholerae* chromosome (Mekalanos *et al.*, 1979; Sporecke *et al.*, 1984). Sinha & Srivastava (1978*a, b*) observed suppression of virulence of *V. cholerae* after acquisition of P and/or V plasmids, which was associated with partial or complete inhibition of toxin biosynthesis. The A subunit of cholera toxin promotes activation of adenylate cyclase by a NAD-dependent reaction (Gill & Meren, 1978). The B subunit of the toxin, also referred to as cholera toxin, may be considered a natural toxoid, stimulating antitoxin immunity (Holmgren *et al.*, 1977). A⁻B⁺ strains attenuated by classical mutagenesis (Honda & Finkelstein, 1979) and by recombinant DNA techniques (Mekalanos *et al.*, 1983; Kaper *et al.*, 1984) have been isolated.

The objective of our study was to cure *V. cholerae* cells of plasmids and to determine whether the loss of plasmid resulted in reversion to a normal level of toxin biosynthesis as in isogenic strains devoid of plasmids.

METHODS

Media. Broth cultures of bacteria were raised in Difco Bacto Brain Heart Infusion (BHI; 37 g in 1 litre distilled water). Bacterial plating was done on Difco nutrient agar plates (NA; 8 g Difco nutrient broth, 5 g NaCl, 10 g Oxoid agar in 1 litre distilled water). Syncase sucrose minimal medium was prepared as described by Finkelstein & LoSpalluto (1969). Phosphate-buffered saline (PBS; pH 7.2) was routinely used for bacterial dilution (Sinha & Srivastava, 1978). An aqueous solution of ampicillin (Ap, 100 µg ml⁻¹) was filter-sterilized and added after the media were autoclaved.

Bacteria. *Escherichia coli* CSH41 harbours the conjugative plasmid RP4 which carries the Ap^r determinant transposon Tn1; this transposon was transposed onto the P plasmid of *V. cholerae* to introduce a genetic marker for plasmid identification. *V. cholerae* CD63 was characterized as harbouring P⁺:Tn1 and Ap^r.

Abbreviations: HA, haemagglutination; SRBC, sheep red blood cells.

V. cholerae CD65 and CD66 harbour the temperature-sensitive plasmid *ts P::TnI* (see below). CD68 is a cured isolate of CD66. It was isolated by growing CD66 at 42 °C and streaking on NA. A single-colony isolate that was *P*⁻ and *Ap*^s was considered cured. CD70 is a *P*⁺ derivative of CD68. It was isolated from a cross between CD63(*P::TnI*) and CD68 and selected on NA + Sm + *Ap*. KB365(*P::TnI*) was isolated from a cross between CD63(*P::TnI*) and KB365 (a spontaneous *Sm*^r mutant of strain 569B Inaba) and selected on NA + Sm + *Ap*.

V. cholerae CD60 is a choleraenoid-producing strain screened from among *P*⁺*V*⁺ attenuated isolates of strain 569B Inaba. The *P* and *V* plasmids were transferred by conjugation from strains KB9(*P*) and KB9(*V*).

Isolation of strains CD65 and CD66, carrying ts P::TnI. The plasmid *ts P::TnI*, whose replication is inhibited at 42 °C but unaffected at 30 °C, was isolated as follows. *V. cholerae* CD63 was grown exponentially to 5×10^8 cells ml⁻¹ in BHI. The bacteria were washed and suspended in sodium citrate buffer (pH 5.5, 0.1 M) containing *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (150 µg ml⁻¹) and shaken for 20 min at 37 °C. The mutagenized bacteria were washed with PBS and suspended in 10 ml BHI containing ampicillin and shaken overnight at 30 °C, then crossed with *V. cholerae* KB365 at a ratio of 1:9 at 30 °C for 90 min. The mating mixture was streaked on NA + Sm + *Ap*, incubated at 30 °C for 12–14 h and examined for small isolated colonies. The plates were then moved to 42 °C for 8 h to select probable *ts* mutants on the basis of small colony size. The tiny colonies with arrested growth at 42 °C were tested on NA + *Ap* at 30 °C, on NA at 42 °C, and on NA + *Ap* at 42 °C. Colonies not growing at 42 °C on one or both were considered to have the *ts* plasmid. (The presence of the *P* plasmid was tested as described below.) Two of these isolates, designated CD65 and CD66, were retained for curing studies.

Test for plasmid carriage. Presence of plasmid was determined by the growth-thinning effect of *P*⁺/*V*⁺ strains on the plasmid-negative strain KB9 (Bhaskaran, 1964). Strain KB9 (0.2 ml, about 2×10^8 cells) was mixed in 7 ml soft agar and poured on a NA plate. A loopful of the test culture was placed on the lawn of KB9 and incubated overnight at 30 °C or 37 °C as specified in the text. In addition, the presence of *P::TnI* and its derivatives was determined by the *Ap*^r character of the bacteria.

Preparation and assay of toxin. Toxin was obtained by growing bacteria in syncase sucrose minimal medium at 28 °C for 16 h in a shaker. Bacteria-free culture filtrate was obtained by membrane filtration (Millipore, 0.22 µm pore size). Toxin antigen was quantified *in vitro* by radial immunodiffusion. Rabbit antitoxin antibodies were mixed in 2% (w/v) agarose gel into which wells of equal diameter were cut. The wells were charged with 20 µl of toxin preparations. With known concentrations of pure toxin, there was a linear relationship between toxin concentration and the square of the diameter of the antigen-antibody reaction zone. Toxin was measured *in vivo* in adult albino guinea-pigs (Craig, 1965). Culture filtrate was diluted in BHI and 0.1 ml was injected intradermally. Induration was recorded after 18 h. BHI alone caused no induration. With known preparations of pure toxin, the diameter of the zone of induration showed a linear relationship to the log of the concentration of toxin, within the range 5–30 mm.

Sensitization of sheep red blood cells (SRBC) by cholera toxin and its A and B subunits. Fresh SRBC were sensitized by the method of Hochstein *et al.* (1970). Briefly, SRBC were washed three times in cold saline (0.85%) and the cells were suspended to a final concentration of 5% (v/v) in pure toxin (15 µg per ml saline) or A or B subunit toxin (each 25 µg per ml saline; all purchased from Calbiochem, USA). The suspension was incubated for 30 min at 22 °C and washed three times in cold saline. Between 80 and 85% cells were sensitized. The extent of sensitization (%) was measured by a complement-mediated lysis test. Antitoxin serum, heated to 56 °C for 30 min to inactivate complement, was absorbed with SRBC to remove naturally occurring antibodies against SRBC. Then the serum (0.3 ml) was incubated with 0.3 ml toxin-sensitized SRBC at 22 °C for 40 min. Cells were washed once and 0.3 ml 10% (v/v) guinea-pig complement was added. As controls, equal amounts of sensitized SRBC were incubated separately with antibody, complement or water. After incubation at 37 °C for 1 h, the absorbance of the cleared supernatants were recorded at 520 nm.

Titration of serum antibody. Antitoxin levels were determined by a passive haemagglutination (HA) test with toxin-sensitized SRBC (Hochstein *et al.*, 1970). Briefly, 0.3 ml of serial dilutions of sera were mixed with 0.3 ml sensitized SRBC in HA plates and incubated for 2 h at 37 °C followed by overnight incubation at 4 °C. Titres were recorded as the highest serum dilution showing positive HA. If no HA activity was detected in a dilution of 1 in 20, the serum was considered to lack antitoxin antibodies.

Immunization of rabbits with culture filtrates. Albino rabbits weighing 1–1.2 kg were immunized by two subcutaneous injections, two weeks apart, of 1 ml bacteria-free culture filtrate. Two weeks after the second dose, blood was withdrawn for serum antibody titration and rabbits were challenged by the ileal loop test to measure protection (Sinha & Srivastava, 1978b).

RESULTS

Characterization of P::TnI

The effect of plasmid *P::TnI* on toxin biosynthesis was compared with that of plasmid *P* by measuring toxin in the culture filtrates of KB365, KB365(*P*) and KB365(*P::TnI*). Toxin

Table 1. Assay of toxin in the culture filtrate of parent P^- , P^+ and cured strains of *V. cholerae*

Strain	Plasmid status	Relevant markers*	Toxin† ($\mu\text{g ml}^{-1}$)
KB365	—	Sm ^r , derivative of 569B Inaba	17.0 $\pm$ 1.2
KB365(P)	P	Sm ^r	1.5 $\pm$ 0.45
KB365(P::TnI)	P::TnI	Sm ^r Ap ^r	1.6 $\pm$ 0.2
CD65	ts P::TnI	Sm ^r Ar ^r , derivative of KB365	1.4 $\pm$ 0.4
CD66	ts P::TnI	Sm ^r Ap ^r , derivative of KB365	1.6 $\pm$ 0.4
CD68	'Cured'	Sm ^r , derivative of CD66	15.75 $\pm$ 1.5
CD70	P::TnI	Sm ^r Ap ^r , derivative of CD68	1.3 $\pm$ 0.4

* Sm^r, streptomycin resistant (500 $\mu\text{g ml}^{-1}$); Ap^r, ampicillin resistant (100 $\mu\text{g ml}^{-1}$).

† Toxin was measured *in vitro* by radial immunodiffusion and *in vivo* in guinea-pigs with purified toxin as standard. The results are means of four replicate *in vitro* experiments, $\pm$ SEM. The *in vivo* test gave closely similar results.

biosynthesis was inhibited by both plasmids, indicating that transposition of TnI onto P did not affect the ability to suppress toxin biosynthesis (Table 1).

Characterization of ts P::TnI

As described in Methods, 2000 small colonies were tested on NA + Ap at 30 °C, on NA at 42 °C and on NA + Ap at 42 °C. Nineteen colonies were temperature sensitive; they fell into two groups. Group I comprised 13 isolates which grew at 42 °C on NA but not on NA + Ap. CD65 is representative of this group. Group II comprised six isolates, characterized by their inability to grow at 42 °C irrespective of medium, i.e. the plasmids had conferred the ts phenotype on the bacteria. CD66 is one of the isolates of this group. It should be remembered that ts plasmids were characterized after transfer to unmutagenized host KB365. This behaviour can be explained if it is assumed that the plasmid has integrated into the chromosome and the plasmid's origin of replication is being utilized. A similar observation, termed integrative suppression, was reported in *E. coli* by Nishimura *et al.* (1971). The 19 isolates were otherwise similar in growth and ampicillin resistance at 30 °C, presence of P plasmid (determined in the thinning of growth test) and suppressed synthesis of toxin.

Curing the bacteria of plasmids

In view of our objective of obtaining a ts P::TnI which could be eliminated at 42 °C without affecting bacterial growth, ts mutants of both groups described above were examined.

Group I. The bacteria were grown at 42 °C in BHI and suitable dilutions were plated on NA and NA + Ap and incubated at 30 °C. No difference was observed in the number of colonies on the two media, and all the colonies were P^+ in the thinning of growth test. This suggested that cells were not cured of plasmids.

Group II. The six isolates of this group could grow at 30 °C but not at 42 °C on any medium. However, after a few subcultures at 30 °C, one of the isolates (CD66) was able to grow at 42 °C in BHI. After growth of strain CD66 at 42 °C for 4 h, suitable dilutions were plated on NA and NA + Ap and incubated at 30 °C. Colonies appeared on NA but not on NA + Ap, suggesting 100% loss of ampicillin resistance. These Ap^s colonies were P^- in the thinning of growth test. Thus 100% curing of CD66 was observed when it was grown at 42 °C in BHI. The cured derivative of CD66 was designated CD68.

Assay of cholera toxin in P^+ and cured strains

Toxin was measured in the culture filtrates of the parent P^- , P^+ and cured strains (Table 1). All the strains are isogenic. While P^+ strains made less toxin than the P^- parent KB365, the quantity of toxin in the filtrates of the cured strain CD68 was similar to that observed for KB365. Re-introduction of the P plasmid into the cured strain resulted in the suppression of toxin biosynthesis (CD70, Table 1).

Fig. 1. Ouchterlony test of preparation from strains 569B and CD60: rabbit-anti-569B-toxin (I), rabbit-anti-CD60-mutant toxin (II), toxin (T), A subunit (A), B subunit (B). (The lines of precipitation between I and A are very faint.)

Table 2. Serum antitoxin titre and protection in rabbits immunized with bacteria-free culture filtrate (CF) of CD60 and 569B

Six animals of the same age were used in each group. Four ileal loops were made in each rabbit; two were challenged with toxin and two with *V. cholerae* 569B Inaba. Hence for each group antitoxin titre is the mean ($\pm$ SEM) of titres obtained independently from six rabbits, whereas the percentage protection data represent means ($\pm$ SEM) of 10–12 loops. Antitoxin titres were measured in sera from rabbits immunized with 3–4 batches of culture filtrate prepared on different days. The protection experiment was done twice, each time with six rabbits in each group.

Vaccine	Antitoxin titre	Challenged with*	Protection against challenge† (%)
Control‡	< 20	569B	0
		Toxin	0
CF of CD60	200 $\pm$ 8	569B	47 $\pm$ 12
		Toxin	60 $\pm$ 13
CF of 569B	800 $\pm$ 0	569B	63 $\pm$ 10
		Toxin	78 $\pm$ 11

* 10^5 colony-forming units of 569B or 10 μ g of pure cholera toxin in ligated ileal loops; both elicited 1.5–1.8 ml fluid (cm loop) $^{-1}$ in control rabbits.

† Percentage protection is calculated as $(x/y) \times 100$, where x is the volume of fluid (ml cm $^{-1}$) in the ileal loops of immunized rabbits, and y is the volume of fluid in the ileal loops of control, non-immunized rabbits.

‡ Sterile culture medium.

Characterization of strain CD60, producing cholera toxin

CD60 is an attenuated P⁺V⁺ mutant of *V. cholerae* 569B Inaba: neither CD60 nor its culture filtrate had any detectable cholera toxin-like activity. As reported earlier (Sinha & Srivastava, 1978a), strain 569B caused accumulation of fluid in the ileal loops of rabbit. When rabbits were immunized with bacteria-free culture filtrate of CD60, antitoxin antibodies were detected in the serum and rabbits were protected from challenge with pathogenic strains or cholera toxin (Table 2). Similar results were obtained with culture filtrate of 569B. These results strongly suggest that CD60 released cholera toxin, which was nontoxic but immunogenic.

A simple Ouchterlony test in 2% (w/v) agarose suggested that the mutant toxin synthesized by CD60 can be characterized as A⁺B⁺ (Fig. 1). Additionally, when SRBC sensitized by the A or the B subunit of cholera toxin were mixed with anti-CD60-toxin antibodies, positive HA was observed only with B-sensitized SRBC.

DISCUSSION

Sinha & Srivastava (1978a) reported attenuation of virulence caused by P and V plasmids in *V. cholerae*, due to production of less toxin. Neither the mechanism inhibiting toxin biosynthesis

nor the effect of loss of plasmid on toxin biosynthesis was known. Considerable insight would be gained if the plasmids could be eliminated from the cells and toxin production examined. With this objective, ts mutants of the P plasmid were isolated whose replication was inhibited at 42 °C.

Strains cured of the P plasmid resumed toxin biosynthesis like the parent strain and were relieved of the suppression caused by the plasmid. Reacquisition of the P plasmid by the cured strain again resulted in suppression. These results support the hypothesis that toxin biosynthesis is regulated by plasmid-borne genes. Moreover, plasmids seem to play a unique regulatory role in toxin biosynthesis of *V. cholerae*. Not only is synthesis of toxin inhibited, but modulation of the cells to produce cholera toxin can be achieved. CD60 is a unique type of vaccine strain that is both attenuated by plasmids and synthesizes a mutant toxin (A⁻B⁺).

Cloning of cholera toxin genes and the genetic mapping of regulatory mutants of *V. cholerae* have demonstrated that these loci are located on the chromosome (Gennaro *et al.*, 1982; Mekalanos *et al.*, 1979, 1983; Kaper *et al.*, 1984). The results reported in this paper strongly suggest that genes regulating toxin biosynthesis in *V. cholerae* are also located on the P plasmid.

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