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Structural organization of cholera toxin gene and its expression in an environmental non-pathogenic strain of *Vibrio cholerae*

UMA BHATTACHARYYA*, AMIT GHOSH and
RANAJIT K. GHOSH
Indian Institute of Chemical Biology, Jadavpur, Calcutta 700 032, India
*Present Address: Department of Biochemistry, University of North Carolina, Chapel Hill, North Carolina 27514, USA

Abstract. Non-pathogenic, environmental strain of *Vibrio cholerae,* ELTOR Ogawa EW6 carries a copy of the cholera toxin gene in its chromosome. Restriction enzyme digestion followed by Southern blot analysis revealed that the structure of the cholera toxin gene in this organism is different from that found in the virulent strains. The *xba*I site which has been found to be conserved in the cholera toxin of the virulent strains examined so far, is absent here. Results of the RNA dot blot analysis indicated that the cholera toxin gene in EW6 is transcribed much less efficiently compared to the cholera toxin gene present in the virulent strain *Vibrio cholerae* classical Inaba 569B.

Keywords: *Vibrio cholerae;* avirulent strain; toxin gene.

Introduction

The role of non-pathogenic strains of *Vibrio cholerae* found in the aquatic environment, is an intriguing question in the epidemiology of the disease cholera. Each year cholera epidemics surface simultaneously in many places in different third world countries. It is widely believed that these epidemics spring *de novo* from aquatic environments. Very little is known about these environmental isolates, excepting that they are unable to cause infection in man and that their non-pathogenic character is quite stable. Attempts have been made to use some of these isolates as live oral vaccine for cholera without much success (Cash *et al.,* 1974; Levine *et al.,* 1982). Recently there has been a resurgence of interest in these strains and it has been found that while the majority of the non-pathogenic, environmental strains lack the structural gene coding for the cholera toxin (*ctx*) (Kaper *et al.,* 1981), there are a few strains including some non-01 *vibrios* which carry copies of toxin gene in their chromosomes (Hanchalay *et al.,* 1985). However, nothing is known about the organization of the toxin gene in the avirulent strains or its expression there.

We report here our work on the non-pathogenic environmental strain *V. cholerae* ELTOR Ogawa EW6 (hereafter designated as EW6), isolated by Dr. S. Mukherjee from a water source in 1958 (Cash *et al.,* 1974). We show that this strain though unable to elaborate any toxin as measured by rabbit ileal loop test or vascular permeability test, even after repeated propagation (Gerdes and Romig, 1975; present work), nevertheless carries a copy of the toxin gene in mys chromosome. The structure of the gene however is different from that found in the virulent strains. We further show that compared to the *ctx* present in the pathogenic strain *V. cholerae* classical Inaba 569B (henceforth designated as 569B), the *ctx* in EW6 is transcribed with much lower efficiency.

Abbreviations used: *ctx,* Cholera toxin; SSC, standard saline citrate; SDS, sodium dodecyl sulphate; CT gene probe, cholera toxin gene probe.

Materials and methods

Bacterial strains

The strains used in this study *viz.* non-pathogenic *V. cholerae* ELTOR Ogawa EW6 (Cash *et al.,* 1974) and the pathogenic strains *V. cholerae* classical Inaba 569B and *V. cholerae* classical Ogawa 154 (Mukherjee 1978) were obtained from Dr. F. K. Bhattacharyya of our Institute. Cultures were maintained under conditions described by Mitra *et al.* (1986).

Media and growth conditions

Cells were grown as described before (Siddiqui and Ghosh, 1983) in nutrient broth containing Bactopeptone (Difco) 10 *g,* Lab-lemco powder (Oxoid) 10 *g,* Sodium chloride 5 *g,* per litre of glass distilled water. The pH of the medium was adjusted to 8· 0. Nutrient agar plates contained 1·5% (w/v) Bactoagar (Difco) in nutrient broth.

Colony Hybridization

This was done essentially according to the method of Grunstein and Hogness (1975). Bacterial colonies from the master plate was transferred to a nitrocellulose disc (Schleicher and Schuell, Keene, N. H., USA). Colonies were lysed *in situ* and the DNA imprint was fixed to the disc by baking. Hybridization to [^{32}P]-labelled cholera toxin gene probe was done as described below. After hybridization the filter was monitored by autoradiography.

Toxin assay

Either whole cell lysates or cell free culture supernatants were used as toxin preparations. Lysate preparation by sonication was essentially according to Burrows *et al.* (1965). The amount of toxin produced by a culture was assayed by (i) the rabbit ileal loop method (Kasai and Burrows, 1966); (ii) the vascular permeability test (Craig, 1971). In rabbit ileal loop test, not more than 100 cm of gut measuring from anterior to appendix was used. Two ml of cell lysates or cell free supernatants was inoculated directly or after suitable dilutions, in to five 10-12 cm loops. Negative (broth) and positive (animal passaged 569B cells) control loops were included in all experiments. Fluid accumulation in ligated loops, after 24 h was taken as a measure of toxicity. Amount of toxin produced was expressed as the ratio of the volume of fluid accumulated to the length of the negative control loop.

In vascular permeability test 0·1 ml of the suitable dilutions of the cell-free culture supernatant was injected intradermally on the depilated back of rabbits. After 24 h, a 5% (w/v) solution of pontamine sky blue 6Bx (Gurr, England) was injected intravenously (1·2 ml per kg body weight). The diameter of the blue lesion was measured 1 h after the injection of the dye. Toxin level was expressed as the blueing dose per ml of culture. One blueing dose is equivalent to a blue lesion of 7 mm diameter (Craig, 1971).

Southern blot analysis

Southern blot analysis was done essentially as described before (Ghosh *et al.,* 1985). Briefly, chromosomal DNA from a given strain was digested to completion with different restriction endonucleases either singly or in combination. The digests were then fractionated by electrophoresis in vertical 1% Agarose gels containing 40 mM Tris (pH 8·3), 20 mM Sodium acetate and 1 mM EDTA. The fragments were denatured and then transferred to nitrocellulose sheets (Schleicher and Scuell, Keene, N. H., USA) by the method of Southern (1975). The highly purified cholera toxin gene probe (kindly provided by Dr. P. J. Greenaway) was labelled with [^{32}P]-by nick translation (Rigby *et al.,* 1977). Hybridization solution consisted of buffer containing 50% Formamide, 5 × SSC, 1 mM EDTA, 1% sodium dodecyl sulphate (SDS), 100 μg/ml sheared calf thymus DNA and 1 × Denhardt's solution (Denhardt, 1966). Hybridization was performed at 37°C for 24–36 h in a sealed plastic bag. Blots were first washed in 5 × standard saline citrate (SSC)– 0·1% SDS for 45 min at 55°C and then in 2 × SSC for 15 min at room temperature. These were then autoradiographed for 3–7 days at — 70°C using intensifying screens.

RNA blot analysis

RNA for the RNA blot hybridization was purified according to Aiba *et al*. (1981), from log phase cells of *V. cholerae,* growing in CYE medium (Mekalanos *et al.,* 1977). 100 μg of purified RNA was dissolved in 400 μl of sterile glass distilled water. To this 1·2 ml of a solution of 6·15 M formaldehyde, 10 × SSC was added. The resulting mixture was heated to 65°C for 15 min and then transferred to a Nitrocellulose sheet using a Minifold II slot blot system (Schleicher and Schuell, Keene, N. H., USA), according to the method recommended by the manufacturer. Hybridization wmyh [^{32}P]-labelled chlora toxin probe was done under reduced stringency condition (25% formamide) according to Thomas (1983).

Results

Examination for the presence of toxin gene (ctx) in V. cholerae EW6

In situ colony hybridization method of Grunstein and Hogness (1975) was used to screen *V. cholerae* EW6 for the presence of DNA sequences homologous to the *ctx* gene. Bacterial colonies were grown on nitrocellulose filter, denatured *in situ* and then hybridized with [^{32}P]-labelled cholera toxin gene probe. As can be seen from the autoradiograph (figure 1), EW6 gave a positive signal indicating that a *ctx like* sequence is present in it. Closer examination of the autoradiograph revealed that while both the virulent strains gave strong positive reaction, the hybridization signal obtained with EW6 was somewhat weaker. Since the hybridization was carried out under highly straingent condition (50% formamide), it indicated that either the *ctx* sequence present in EW6 is closely homologous but probably not identical to those found in the toxinogenic strains or that only a single copy of *ctx* is present in EW6, while the virulent strains carry more than one copy of the *ctx* gene.

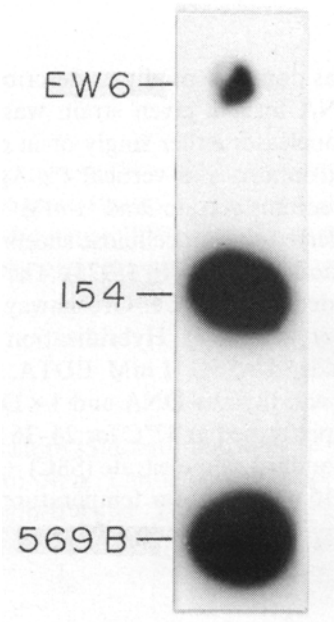

Figure 1. Colony-hybridization of pathogenic and non-pathogenic strains of *V. cholerae* with CT gene probe.

Enterotoxinogenecity of EW6 after animal passage

It has been reported in the literature that the non-pathogenic character of the environmental strain EW6 is quite stable (Gerdes and Romig 1975). In view of our observation that the strain EW6 carries a sequence closely homologous to the *ctx* gene. We thought it necessary to reconfirm this observation. The result of our experiment is summarised in table 1. As can be seen, no detectable amount of toxin was produced in EW6. In contrast the strain 569B exhibited a marked increase in toxin production.

Table 1. Toxin production by *V. cholerae* strains before and after animal passage.

	Toxin production measured			
	Fluid accumulation in ileal loop ml/cm		Blueing dose/ml	
Status	EW6	569B	EW6	569B
Before animal passage	0	0·48 ± 0·15	0	650 ± 55
After first animal passage	0	1·52 ± 0·25	0	1600 ± 125
After second animal passage	0	1·64 ± 0·20	0	2100 ± 75

Each value represents mean of 5 independent determinations.

Structural characteristics of the ctx gene in E W6

We examined the molecular structure of the toxin gene present in EW6 by southern blot analysis. DNA extracted from EW6 was digested with different restriction

enzymes, fractionated by electrophoresis, transferred to nitrocellulose membrane and then hybridize with *ctx* probe under high stringency condition. Analysis of the well characterized *ctx* gene from the strain 569B was carried out side by side for comparison (figure 2). Several interesting differences in the digestion pattern was observed. Strain 569B is known to carry a toxin gene duplication and yields two bands on Southern blot when analysed with *Xba* I, Pst I, *Bgl* II and *Eco*RI. *Xba* I smye is known to be conserved among several cholera toxin genes derived from both classical and ELTOR strains (Gennaro *et al.,* 1982; Gennaro and Greenaway, 1983; Mekalanos *et al.,* 1983). Moreover in all ELTOR strains which contain only a single copy of the cholera toxin operon, *ctx* is found to be located on *Xba* I fragments of 11 kb or less (Mekalanos, 1983). In contrast, in EW6 it was found to be located on a fragment larger than 23 kb in size, indicating that in EW6, the Xbal site is not conserved. When the analysis of EW6 DNA was carried out with *Bgl* II, *Pst* I and *Hind* III in each case it produced two bands of unequal intensity on the Southern blot (figure 2). This was found to be a highly reproducible pattern and could not be an artefact. The only way to interpret this data it seems, is to assume that *ctx* in EW6 is not duplicated (for if it were then one would expect the two bands to be of equal intensity) but rather that these enzymes cleave EW6 *ctx* assymetrically giving rise to one large fragment spanning most of the *ctx* and one small fragment, and it is this smaller fragment which gives rise to the weaker band on the autoradiograph. It is of interest to note here that all the *V. cholerae* ELTOR strains examined so far give rise to only one band on Southern blot when *Hind* III is used for analysis. Double digestion patterns also revealed interesting differences between the *ctx* of 569B and that of EW6. Digestion of 569B chromosomal DNA with XbaI and Hinc II is known to release a 1 kb fragment (figure 2, Mekalanos *et al.,* 1983) from the *ctx* gene. This however was not observed with the *ctx* present in EW6 (figure 2). It is worthmentioning here that *Xba* I + *Hinc* II digestion of chromosomal DNA of ELTOR 1621 a virulent strain and the only one ELTOR strain on which this particular double digestion analysis has been done also releases a 1 kb fragment (P. J. Greenaway, personal communication).

Quantitation of toxin gene specific mRNA by slot blot analysis

The presence of the toxin gene in EW6, though of a different structure, and the absence of toxin production as determined by rabbit ileal loop assay and vascular permeability test, prompted us to examine the expression of this gene *in vivo.* To quantitate the amount of toxin gene specific mRNA, if at all made, in this strain, we performed an RNA dot blot analysis as described in 'materials and methods'. It can be seen from figure 3 that a much lower level of *ctx* gene specific mRNA is made in *V. cholerae* EW6 compared to that in the virulent strain 569B.

Discussion

The data presented in this paper show that the environmental non-pathogenic strain of *V. cholerae,* ELTOR Ogawa EW6, even though phenotypically non-toxinogenic in character, nontheless carries a copy of the toxin gene in its chromosome. The *ctx* gene present in this organism, however, has a structure different from that found in its pathogenic cousins. RNA blot analysis revealed that in contrast to the *ctx* present in

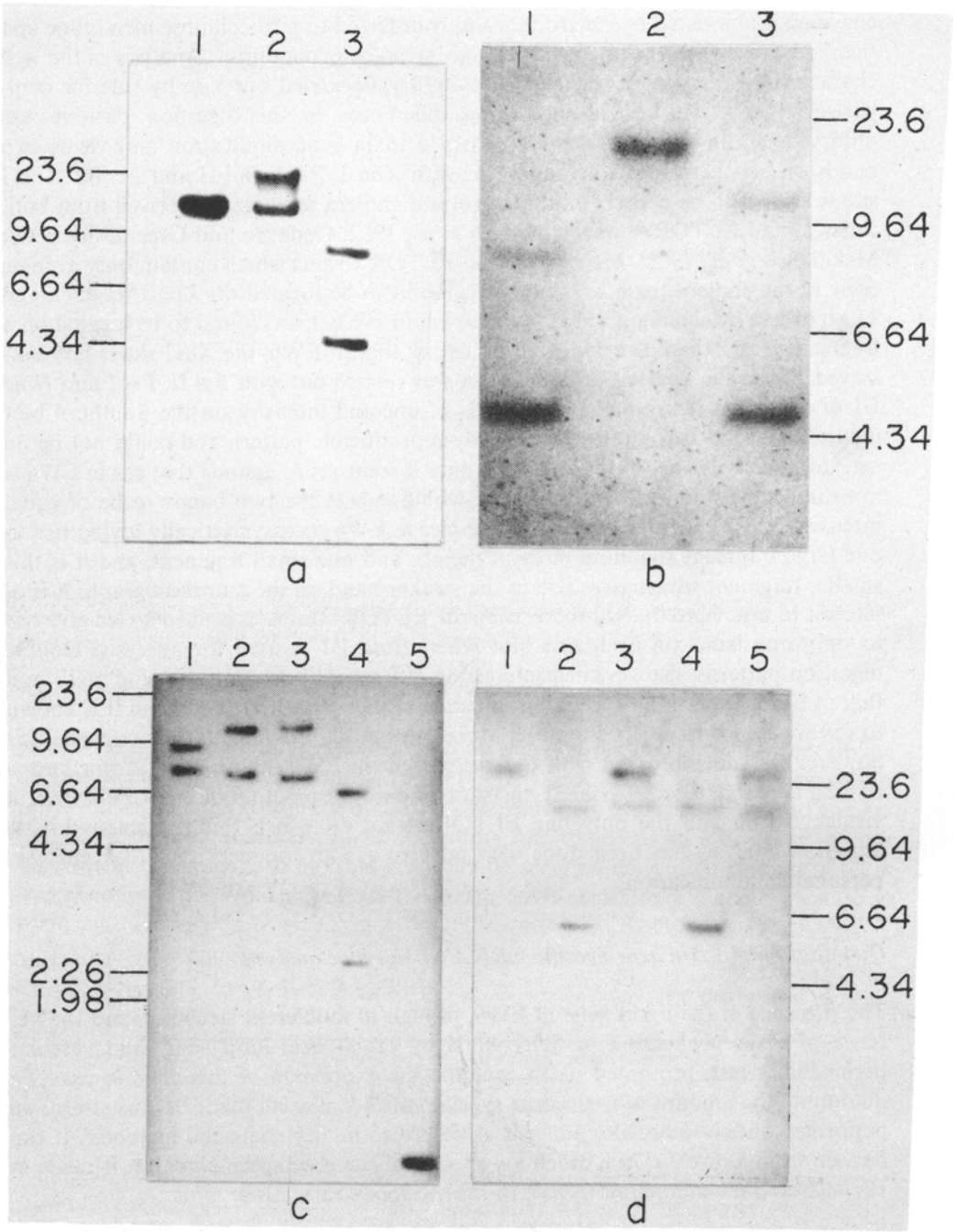

Figure 2. Southern blot analysis of *V. cholerae* DNA derived from strain 569B (a and c) and EW6 (b and d) using [^{32}P]-labelled toxin gene probe. DNA (1 μg) from each strain was digested with various enzymes singly or in combination, fractionated by electrophoresis on agarose gel, blotted and hybridized with nick translated CT gene probe. Lanes 1-3 (a and b) represent digestion with *Hind III, EcoRI* and *Pst I.* Lanes 1-5 (c and d) show digestion with *Xba I, Bgl II, Hind II, Xba I + Bgl II* and *Xba I + Hind II,* respectively. Positions of λ DNA /*Hind III* fragments (kb) run on the same gel are indicated by arrows.

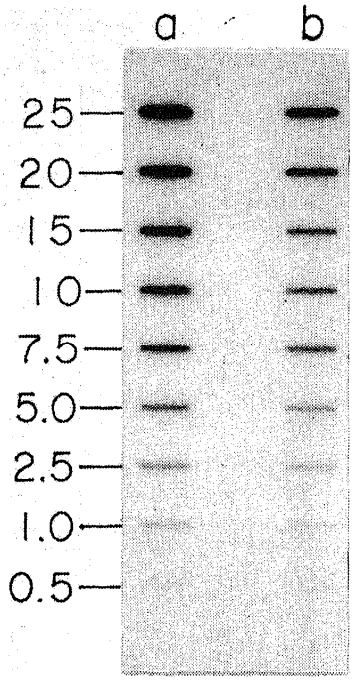

Figure 3. Slot blot analysis of the toxin gene transcripts synthesized by *V. cholerae* strain 569B (a) and EW6 (b). Total RNA was isolated from both the strains as described in 'materials and methods'. This was then denatured and immobilized on nitrocellulose membranes. The filter was then hybridized with nick translated CT gene probe. The amount of RNA (μg) loaded per slot in either case is indicataed in the margin.

the virulent strain 569B, *ctx* in EW6 is transcribed with reduced efficiently. Why this is so is not clear at the moment. It could be because of some defect in the putative toxin regulatory gene *(tox R)* found to be present in all *V. cholerae* strains examined so far or it could be because of some abberation in the promoter region of the *ctx* itself (Miller and Mekalanos 1984). Another unsolved riddle is the apparent non-toxinogenic phenotype of EW6 despite the presence of *ctx* and its expression in it. It may be that the reduced amount of toxin it makes, is not enough to confer patho genecity on it or it could be because of the changed sequence of the *ctx* gene is EW6, the toxin it produces is defective and hence non-functional. Investigation is currently under way to distinguish between these two possibilities.

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