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The impact of physico-chemical stress on the toxigenicity of *Vibrio cholerae*

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SUMMARY

The expression of toxigenicity by *Vibrio cholerae*, before and after exposure to various conditions of salinity, pH and cation composition and concentration, has been measured. Exposure to these conditions did not select for hyper- or hypo-toxigenic strains. This suggests that toxigenic *V. cholerae* O1 are unlikely to lose their toxigenicity when exposed to environmental stress and that *V. cholerae* toxin production is not a response to the stresses included in this study. These results are consistent with an aquatic reservoir for toxigenic *V. cholerae* O1.

INTRODUCTION

In a recent paper (Miller, Drasar & Feachem, 1984), we have reported on the survival of toxigenic *Vibrio cholerae* O1 in water under various conditions of salinity, pH, temperature and cation concentration and composition. The results indicate that toxigenic *V. cholerae* O1 are able to survive for extended periods (months) in warm water (25 °C) containing no nutrients but having a salinity of 0·25–3·0% and a pH of around 8·0. With added nutrients and under the same conditions, rapid growth is possible.

These findings provide further evidence for an aquatic reservoir for toxigenic *V. cholerae* O1 (Miller, Feachem & Drasar, 1985), provided that environmental stress does not select for non-toxigenic or hypotoxigenic strains. The present study investigates the effect of environmental stress on the toxigenicity of *V. cholerae* O1.

MATERIALS AND METHODS

The experiments in this study were all of similar design. Strains of *V. cholerae* of known toxigenicity were added to a measured volume of suspending fluids having various known chemical compositions. After a defined period, the viable strains were recovered and tested for toxin production. The experimental details have already been described fully (Miller, Drasar & Feachem, 1984).

Experiment on 6 Bangladeshi strains

Six Bangladeshi isolates of *V. cholerae* O1 (CT^+), El Tor, Inaba, three from clinical cholera and three from polluted water, were used for these experiments. Each strain was suspended in 500 ml of each of the suspending solutions listed below. The standardized technique used for inoculating the bottles gave a mean initial count of 5·2 $\log_{10}$ c.f.u./ml with a standard deviation of 0·2. Organisms surviving for 64 days in suspension at room temperature (about 25 °C) in the dark were subcultured. 1·0 ml aliquots of overnight peptone cultures of the organisms were mixed with 0·1 ml glycerol and stored at −70 °C for subsequent toxin assay. Replicate cultures of each parent strain were also prepared in the same manner.

The following suspending solutions, all based on autoclaved distilled water, were used: sea salt solutions of the following concentrations (%): 0·05, 0·10, 0·25, 0·50, 1·0, 2·0 and 3·0; 1·0% NaCl solutions with pH adjusted to 6·0, 6·5, 7·0, 7·5, 8·0, 8·5 and 9·0 with di-sodium tetraborate/potassium di-hydrogen phosphate buffer (osmolarity 340 mOsmol); solutions of osmolarity 340 mOsmol and pH 8·0 made with NaCl, KCl, KCl+NaCl, $CaCl_2$+NaCl, $MgCl_2$+NaCl and $SrCl_2$+NaCl; solutions of (%) 0·001, 0·01, 0·10, 1·0, 2·0 NaCl with osmolarity adjusted to 340 mOsmol with KCl and at pH 8·0.

Experiment on 27 strains

Twenty-seven strains of *V. cholerae* were suspended in 500 ml of 0·05% sea salt at pH 8·0 and stored in the dark at room temperature (about 25 °C). This solution provides some osmotic support for *V. cholerae* whilst still being of potable salinity. They were removed from this solution after 16 days.

Experiment on 13 strains

Thirteen strains of *V. cholerae* O1 El Tor, 8 from clinical cholera and 5 from water, were each suspended in 500 ml of a simple salt solution with the following composition: $(NH_4)_2SO_4$ 0·1 g, NaCl 0·5 g, $MgSO_4.7H_2O$ 0·02 g, K_2HPO_4 0·1 g, distilled water 100 ml. The pH of the solution was 8·0. This solution represents favourable conditions for survival of *V. cholerae* and supports the long-term survival of these organisms. The strains were removed from the solution after 40 months in darkness at room temperature (about 25 °C).

Cholera toxin assays

The test strains of *V. cholerae* were grown aerobically in 25 ml conical flasks containing 5 ml syncase medium (Finkelstein *et al.* 1966) at 30 °C in a shaking water bath. After 18 h the cultures were centrifuged at 4500 *g* for 20 minutes. 1·0 ml of supernatant was removed and stored at −70 °C. Cholera toxin was assayed by tissue culture and enzyme-linked immunosorbent assay (ELISA).

Tissue culture. African Green Monkey kidney cells (Vero cells) were used in the method described by Giugliano, Mann & Drasar (1982). Serial dilutions of culture supernatant were mixed with freshly seeded Vero cells and the cell monolayers examined after 18 h incubation. Results are expressed as the reciprocal of the highest dilution of culture supernatant causing > 50% of the cells in the monolayer to give a cytotonic response.

ELISA. A modification of the method of Sack *et al.* (1980) was used, with GM_1 ganglioside as the primary antigen binding material. The modifications made to the technique were that rabbit anti-cholera toxin and an oxidase enzyme system were used. Results are expressed as corrected absorbances which are the test absorbances minus the negative control, multiplied by a factor to correct for the variation which occurs among different microtitre plates. The values were calculated as follows:

$$\text{corrected absorbance} = \left[\text{test absorbance} - \text{absorbance of negative control}\right] \times \left[\frac{\text{lowest value, positive-negative control absorbance of all plates tested}}{\text{test-plate positive control absorbance} - \text{test-plate negative control absorbance}}\right]$$

RESULTS

The following results show that the two different test methods employed in the toxin assays often show different changes in toxin production between parent and test strains. This is thought to be due to differences in the parameters measured. The tissue culture method measures active cholera toxin whilst the ELISA method measures the presence of toxin proteins and will, for example, give a positive result if only dissociated B subunits of cholera toxin are present. It is assumed that if any selection for hyper- or hypo-toxigenic strains were occurring, both tissue culture and ELISA cholera toxin titres would be either raised or lowered.

Toxin production response of 6 strains

Results from the toxin assays of six strains exposed for 64 days to different salinities, pHs, cation combinations and sodium concentrations are given in Tables 1 and 2. The data were analysed by a modification of Wilcoxon's rank sum test. The ELISA value describing the cholera toxin content of each test culture supernatant and the 4 values describing the cholera toxin content of 4 replicate culture supernatants of the parent strain were ranked from 1 to 5 (Table 1). The ranks of the test isolates surviving any one stress were then summed. If there were no gain or loss in toxin production the expected rank for each strain would be 3 and so, if six strains survived the stress, the expected sum would be 18 (6×3). With the tissue culture assay, five replicate values describing the toxin production of the parent strain were obtained and each test culture was ranked from 1 to 6 (Table 2). The expected rank was therefore 3·5 and the expected rank sum 21. A standard normal deviate (SND) value in Tables 1 and 2 shows whether the deviation of the observed rank sum from the expected value is greater than would be expected by chance. A positive SND indicates that the rank sum was above the expected value (possible increase in toxin production) while a negative SND indicates that the rank sum was below the expected value (possible decrease in toxin production). A value of $< -1{\cdot}96$ or $> +1{\cdot}96$ shows that on average there was a significant ($P < 0{\cdot}05$) decrease or increase, respectively, in cholera toxin production of the stressed strains compared with the parent strains. If one or more strains failed to survive the stress, the significance test was modified appropriately to include just those strains which survived.

The ELISA results (Table 1) show a statistically significant ($p < 0{\cdot}05$) fall in

Table 1. *The toxin production of six parent and stressed strains of* V. cholerae *O1 as measured by ELISA*

Stress‡	Strain number† BW 150	BW 151	BW 152	BC 158	BC 159	BC 162	SND of rank sum§
	(Corrected absorbance at 492 nm)						
Replicate values	0·06	0·06	0·04	0·06	0·07	0·09	—
of unstressed	0·08	0·07	0·05	0·06	0·08	0·12	—
parent strain	0·09	0·11	0·05	0·07	0·09	0·12	—
	0·10	0·13	0·06	0·13	0·10	0·14	—
Stress exposure							
0·05 % sea salt	—	—	—	—	0·09	—	0·00
0·10 % sea salt	—	—	0·07	0·09	—	0·08	+0·20
0·25 % sea salt	0·31	0·09	—	0·07	0·09	0·01	+0·16
0·50 % sea salt	0·07	0·13	0·07	0·06	0·03	0·08	−0·87
1·0 % sea salt	0·10	0·09	0·06	0·02	0·02	0·02	−1·01
2·0 % sea salt	0·07	0·08	0·10	0·05	0·01	—	−0·63
3·0 % sea salt	0·12	0·11	0·05	0·03	0·03	—	−0·63
pH 6·0	—	—	—	—	0·09	—	0·00
pH 6·5	0·04	—	0·06	0·12	0·14	0·13	+0·95
pH 7·0	0·04	—	0·06	0·15	0·06	0·13	0·00
pH 7·5	0·06	0·11	0·06	0·11	0·09	0·14	+0·87
pH 8·0	0·93	0·03	0·10	0·11	0·10	0·15	+1·73
pH 8·5	0·11	0·04	0·09	0·09	0·13	0·11	+1·01
pH 9·0	0·04	0·05	0·09	0·09	—	0·09	−0·95
NaCl	0·03	0·01	0	0·05	0·03	0·08	−3·61***‖
KCl	—	0·07	0·02	0·01	—	0·03	−2·47*
KCl+NaCl	0·02	0·01	0·03	—	—	0·03	−3·00**
$CaCl_2$+NaCl	0·02	0	0·19	0·03	0·15	0·03	−1·30
$MgCl_2$+NaCl	0·01	0	0·17	0·05	0·09	0·05	−1·73
$SrCl_2$+NaCl	—	—	—	0·08	0·17	0·09	+0·41
0·001 % NaCl	—	—	0·08	—	—	0·03	+0·13
0·01 % NaCl	0·03	0·05	—	0	—	0·02	−3·00**
0·10 % NaCl	0·06	0·03	0·02	0·05	—	—	−2·83**
1·0 % NaCl	0·03	0·01	0·29	—	0·08	0·04	−1·58
2·0 % NaCl	0·04	0·05	0·01	0·12	0·07	0·01	−2·60**

† Key to strain prefix: B denotes strains from Bangladesh, W denotes strains isolated from water, C denotes strains isolated from clinical cholera.

‡ All stressed strains were exposed to the stated conditions for 64 days at 25 °C.

§ see text for explanation.

‖ * $P < 0·05$, ** $P < 0·01$, *** $P < 0·001$.

toxin production in the NaCl, KCl, KCl + NaCl, 0·01 % NaCl, 0·10 % NaCl and 2·0 % NaCl experiments, but no significant change under the other stresses examined. The tissue culture results (Table 2) show a significant rise ($P < 0·05$) at pH 6·5 but no significant change ($P > 0·05$) under any other stress. No significant rise or fall in toxin production, as measured by both ELISA and tissue culture, was seen for any one stress condition.

Table 2. *The toxin production of 6 parent and stressed strains of* V. cholerae *O1 as measured by tissue culture*

Stress‡	Strain number† BW 150	BW 151	BW 152	BC 158	BC 159	BC 162	SND of rank sum‖
	(Cholera toxin titre§)						
Replicate values	16	48	4	<4	0	32	—
of unstressed	64	128	4	4	6	128	—
parent strain	96	128	8	4	32	128	—
	96	128	12	8	32	> 128	—
	128	> 128	12	12	48	> 128	—
0·05% sea salt	—	—	—	—	32	—	+0·35
0·10% sea salt	—	—	16	64	—	8	+0·82
0·25% sea salt	> 128¶	8	—	< 32‡‡	< 32	—	+0·53
0·50% sea salt	32	128	32	64	64	64	+1·16
1·0% sea salt	128	8	32	< 32	16	32	+0·43
2·0% sea salt	12	4	96	16	64	—	+0·63
3·0% sea salt	64	48	64	< 16	64	—	+0·79
pH 6·0	—	—	—	—	128	—	+1·41
pH 6·5	24	—	64	96	128	> 128	+2·21*§§
pH 7·0	2	—	64	64	128	128	+1·27
pH 7·5	24	64	< 64	< 16	128	128	−0·29
pH 8·0	—	128	< 64	< 8	128	> 128	+1·74
pH 8·5	64	64	128	< 32	128	> 128	+1·73
pH 9·0	96	96	64	128	—	> 128	+1·58
NaCl	32	64	< 64	< 8	16	48	−1·01
KCl	—	—	< 16	< 8	—	128	−0·61
KCl+NaCl	8	< 2	< 64	—	—	128	−1·24
$CaCl_2$+NaCl	96	96	< 16	< 8	> 128	< 4	−0·58
$MgCl_2$+NaCl	< 4	16	128	< 16	> 128	32	−0·43
$SrCl_2$+NaCl	—	—	—	64	> 128	128	+1·63
0·001% NaCl	—	—	—	32	—	2	−0·25
0·01% NaCl	96	32	—	4	—	96	−1·59
0·10% NaCl	32	96	16	32	—	—	−0·53
1·0% NaCl	48	64	48	—	> 128	—	+0·88
2·0% NaCl	64	32	4	32	64	24	−0·87

† Key to strain prefix: B denotes strains from Bangladesh, W denotes strains isolated from water, C denotes strains isolated from clinical cholera.

‡ All stressed strains were exposed to the stated conditions for 64 days at 25 °C.

§ The reciprocal of the highest dilution of culture supernatant which produced a cytotonic response in > 50% of the cells in the Vero cell monolayer to which it was exposed.

‖ See text for explanation.

¶ When the 50% end point was above the highest dilution used (1/128) the results were expressed as > 128 and analysed statistically as 256.

‡‡ Some culture supernatants exhibited cytotoxicity to Vero cells. If, for example, the plates were unreadable at 1/2, 1/4, 1/8, and showed less than 50% cells affected at 1/16, the result was recorded as < 1/16 and analysed statistically as 1/8.

§§* $P < 0{\cdot}05$.

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Table 3. *The toxin production of 27 strains of* V. cholerae *before and after 16 days exposure to 0·05% sea salt at 25 °C*

Strain† number	ELISA assay of toxin (Corrected absorbance at 492 nm)		Tissue culture assay‡ of toxin (cholera toxin titre)	
	Before exposure	After exposure	Before exposure	After exposure§
TW1	0·11	0·09	128	128
TW3	0·03	0·05	32	< 4
TW8	0·02	0·02	0	0
TW9	0	0·05	32	< 4
TC11	0·08	0·27	64	> 128
TC13	0	0	< 64	0
TC14	0·07	0	32	8
TC16	0·02	0·04	< 2	32
TC17	0·06	0·09	128	8
TC18	0·07	0·02	> 128	24
TC23	0·05	0·04	64	128
TC25	0·05	0	96	128
TW27	0·17	0·08	> 128	64
TW33	0·03	0·01	0	0
BC166	0·10	0·17	128	**
BC161	0·11	0·04	> 128	128
BC164	0·11	0·12	< 128	64
BC165	0·08	0·12	> 128	**
BC169	0·07	0·14	> 128	**
BC170	0·06	0·18	64	**
BC173	0·04	0·22	> 128	**
BW200	0	0	< 4	0
BW202	0	0	0	0
BW203	0	0	0	0
BW204	0	0	0	0
BW205	0	0·07	0	0
AW251	0·01	—‖	< 16	< 4

† Key to strain prefix: B denotes strains from Bangladesh, T denotes strains from Tanzania, A denotes strains from Australia, W denotes strains isolated from water, C denotes strains isolated from clinical cholera. All Bangladeshi water isolates were of non-O 1 serogroups, all other strains were of the O 1 serogroup.

‡ See footnotes §, ¶, ‡‡, Table 2.

§ Tissue culture titres are not given for all supernatants. In these supernatants (**) the titre of the cytotoxic effect was so high that no cells were seen exhibiting a cytotonic effect.

‖ Results technically inadequate.

Toxin production response of 27 strains

Results from the toxin assays of 27 strains exposed to 0·05% sea salt for 16 days at 25 °C are presented in Table 3. The results were analysed by the paired t test. Results for those strains showing no toxin production before and after stress, in both ELISA and tissue culture assays, were omitted from the analysis. The ELISA assay showed a mean rise in absorbance between parent and stressed isolates of 0·022, though this was not statistically significant ($t = +1·39$, 21 d.f., $P > 0·05$). The data from the tissue culture assay were analysed in the same way, though the

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Table 4. *The toxin production of 13 strains of* V. cholerae *O1 before and after 40 months suspension in a simple salt solution*

Strain number†	ELISA assay of toxin (corrected absorbance at 492 nm)		Tissue culture assay‡ of toxin (cholera toxin titre)	
	Before exposure	After exposure	Before exposure	After exposure
TW1	0·11	0·04	128	> 128
TW2	0·04	0·01	128	96
TW3	0·03	—§	3	> 128
TW8	0·02	0·01	**	**‖
TW9	0	0·01	32	< 128
TC11	0·08	0·01	64	> 128
TC12	—	—	6	< 64
TC13	0	0·01	< 64	< 4
TC14	0·07	0·02	**	**
TC15	—	—	64	64
TC103	0·05	0	**	**
TC110	0	0·03	**	**
TC112	0	0·03	**	**

† Key to strain prefix: T denotes strains from Tanzania, W denotes strains isolated from water, C denotes strains isolated from clinical cholera.

‡ See footnotes §, ¶, ‡‡, Table 2.

§ Results technically inadequate.

‖ Tissue culture titres are not given for all supernatants. In these supernatants (**) the titre of the cytotoxic effect was so high that no cells were seen exhibiting a cytotonic effect.

figures used for the analysis were not the filtrate titres given in Table 3 but the $\log_2$ titres; for example, titres of 2, 4, 8 and 16 were analysed as 1, 2, 3 and 4 respectively. This ensured that the analysis would not be dominated by falls or rises in toxin production occurring at the high dilution end of the dilution range. Using this method of analysis there was a mean fall in toxin production between parent and test isolates of 1·11 log intervals. This was not statistically significant ($t = -1{\cdot}88$, 17 d.f., $P > 0{\cdot}05$).

Toxin production response of 13 strains

Results from the toxin assay of 13 strains exposed to a simple salt solution for 40 months at 25 °C are presented in Table 4. They were analysed by the paired t test. Neither ELISA ($t = -1{\cdot}60$, 9 d.f., $P > 0{\cdot}05$) nor tissue culture ($t = +1{\cdot}01$, 7 d.f., $P > 0{\cdot}05$) showed a significant rise or fall in toxin production.

Toxin response of all strains

Results from Tables 1 and 2, and 3 and 4 were combined to measure the impact of non-specific stress, such as suspension in nutrient-free conditions, on *V. cholerae* toxigenicity. Three of the 50 changes in toxin production recorded in Tables 1 and 2 showed no change, 23 showed an increase in toxin production and 24 showed a decrease. Four of the 55 paired cholera toxin titres shown in Tables 3 and 4 showed no change, 25 showed an increase in toxin production and 26 a decrease. There

was no generalized rise or fall in toxin production after stress exposure as measured by either set of data.

DISCUSSION

The role of cholera toxin production in *V. cholerae* O1 is central to the current debate on the ecology of these organisms. The toxin may be produced to cause diarrhoea in man or it may be produced to enable the organisms to exploit a given environmental habitat and only cause diarrhoea in man incidentally.

Results of this study do reveal something of the role of cholera toxin in the ecology of *V. cholerae* (CT^+). The failure to demonstrate an association between stress resistance and high toxin production suggests that toxin production is not a response to any of the stresses included in the study. If it were, the selective pressure of the stress would be expected to select for test strains that produce more toxin than the parent strain.

The lack of an association between exposure to low salinity and high toxin production is of particular interest. Until comparatively recently, the only *V. cholerae* O1 known were those that produced cholera toxin. These were almost exclusively isolated from clinical cases and low salinity waters in association with cases of clinical cholera. *V. cholerae* O1 have now been isolated from the environment without being associated with clinical cholera. These strains are frequently isolated from waters of comparatively high salinity and many of them do not produce cholera toxin, thus it might be suggested that cholera toxin may be produced to enable *V. cholerae* to inhabit low salinity waters. The result that cholera toxin production is not influenced by or associated with low salinity suggests that water salinity is not directly responsible for the differential isolation of toxin and non-toxin producing *V. cholerae* O1 from low or high salinity water.

The absence of an association between stress resistance and high levels of toxin production does not mean, however, that cholera toxin is not produced to overcome a particular environmental stress. The toxin gene may be an adaptation to a stress not included in this study and only encountered in specific micro-habitats within the aquatic environment. This possibility gains support from the demonstration that many non-O1 *V. cholerae* which do survive in the environment (Craig *et al.* 1981), some of the *V. cholerae* O1 (CT^-) from the environment (Sanyal *et al.* 1983), and also bacteria belonging to different genera (Jiwa, Krovacek & Wadstrom, 1981), produce proteins with activities very similar to cholera toxin.

Results of this study have also shown little evidence of any loss in the toxin-producing ability of *V. cholerae* O1 (CT^+) when exposed to conditions of low salinity, adverse pH, adverse water chemistry, low sodium or long-term starvation. This suggests that *V. cholerae* O1 (CT^+) are unlikely to lose their toxin-production ability when exposed to environmental stress. If *V. cholerae* O1 (CT^+) are able to survive in the environment, their retention of toxigenicity means that an aquatic reservoir may be the mechanism responsible for the maintenance of endemic cholera (Miller, Feachem & Drasar, 1985).

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