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Response of toxigenic *Vibrio cholerae* 01 to physico-chemical stresses in aquatic environments

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SUMMARY

The survival and growth of toxigenic *Vibrio cholerae* 01 in water under various conditions of salinity, pH, temperature and cation composition and concentration were studied in an extensive series of laboratory experiments. Inter- and intra-strain variation in stress response (of 01 and non-01 strains) and the ability of *V. cholerae* to adapt to stressful environments were also studied. Toxigenic *V. cholerae* 01 were able to survive for at least 70 days at 25 °C in solutions of sea salt. The optimal salt concentration was 2·0 % though all solutions in the range 0·25–3·0 % gave good support. Substrains with enhanced capacity to persist at sub-optimal salinity (0·1 %) were demonstrated. A great degree of inter-strain variation in stress response at low salinity (0·05 %) was found among 59 strains, and this variation was unrelated to serogroup (01 or non-01), source (clinical or environmental) or country of origin (Tanzania or Bangladesh). At optimal salinity, inter-strain variation was less and 18 out of 20 strains remained viable at high concentrations for at least 40 months at 25 °C. *V. cholerae* 01 could not survive beyond 45 days at 4 °C and optimal salinity, either with or without nutrients. The optimal pH range for survival at 25 °C was 7·0–8·5 at optimal salinity, and 7·5–9·0 at low salinity. *V. cholerae* 01 require Na^+ for survival in the absence of nutrients, and for enhanced growth in their presence. The presence of Ca^{2+} or Mg^{2+}, in addition to Na^+, further enhanced survival. These, and other results reported in this paper, suggest that toxigenic *V. cholerae* 01 are able to survive for extended periods in warm water 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. The implications of these findings for the identification of putative aquatic reservoirs of *V. cholerae* 01, and for the epidemiology of cholera, are considerable.

INTRODUCTION

Cholera toxin producing (CT^+) *Vibrio cholerae* 01 seasonally disappears from both the human population and the environment in some cholera endemic areas (Martin *et al.* 1969; McCormack *et al.* 1969). Four models are available to explain the maintenance of endemicity in these circumstances: carrier status in animals, carrier status in man, continuous transmission in man and an environmental

reservoir. The evidence available suggests that the first three of these models are unlikely.

Toxigenic *V. cholerae* 01 have been isolated from domestic animals only in the locality of current cholera cases in man (Sanyal *et al.* 1974). The evidence for chronic carriage in man is also weak. Only one chronic carrier of cholera has been recognized (Azurin *et al.* 1967). Some convalescent cholera patients excrete cholera vibrios for a few months after infection, though these strains are normally rough (Pierce *et al.* 1970) and are probably non-pathogenic. The model of continuous transmission in man has gained some support because the organism frequently causes sub-clinical infections and continuous transmission could remain undetected. However, continuous transmission of the organism from man to man is unlikely to be a successful strategy because of the comparatively high infectious dose of the vibrio (Hornick *et al.* 1971), the sensitivity of the vibrios to dehydration (Koch, 1884), the adaptation of the organism to waters of high salinity, its limited survival in potable water (Miller, Drasar & Feachem, 1982), and the sensitivity of the organism to acid conditions (Pollitzer, 1959).

An environmental reservoir for *V. cholerae* 01 (CT^+) was suggested by early bacteriologists (Koch, 1884) but was subsequently rejected, mainly because most survival studies have shown that the organisms have only a limited potential for environmental survival (Felsenfeld, 1974; Pollitzer, 1959). However, a number of strains have recently been isolated from the environment in circumstances which suggest that they may be long-term inhabitants of certain aquatic environments (Blake *et al.* 1980; Rogers *et al.* 1980). These isolations have been made in areas where a few cases of clinical cholera have been detected, and the possibility that they may derive from undetected infections cannot be excluded.

The ability of *V. cholerae* 01 (CT^+) to utilize substrates such as chitin that are found in the environment (Nalin *et al.* 1979), and to grow under a range of simulated aquatic conditions (Hood & Ness, 1982; Singleton *et al.* 1982*a*, *b*), suggest that the organisms may be able to grow in estuaries and similar environments. If *V. cholerae* 01 (CT^+) are to survive in the aquatic environment they must be able not only to grow and compete with other organisms for nutrients but also to persist through periods when nutrients are not available. The present study is a laboratory investigation of the response of *V. cholerae* 01 (CT^+) to physico-chemical stresses in water when deprived of nutrients.

MATERIALS AND METHODS

Most of the experiments in this study are similar in design and include the addition of a number of organisms to a measured volume of suspending fluid and the subsequent monitoring of viable numbers over a defined period of time. A standardized technique developed for these experiments is described below.

Organisms used in the study

Six Bangladeshi isolates of *V. cholerae* 01 (CT^+), El Tor, Inaba were used for most experiments. Three of these isolates were from cases of cholera and three were from polluted water. Toxin production was confirmed by a Vero cell tissue culture assay (Speirs, Stavric & Konowalchuk, 1977) and by enzyme-linked immunosorbent assay using GM_1 ganglioside (Sack *et al.* 1980).

Preparation of inoculum

The strains were first inoculated on to trypticase soy agar (BBL) plates and incubated at 37 °C. A loopful of the resulting growth was resuspended in 100 ml 1 % NaCl at pH 8·0. This was vigorously mixed to break up clumps of cells and then allowed to stand for 1 h to enable any remaining clumps to settle.

Preparation and inoculation of suspending fluid and storage of bottles

Glass-distilled water, adjusted to a pH of 8·0 with 0·1N NaOH and sterilized by autoclaving (121 °C, 15 lb/in², 20 min), was the base for most suspending fluids. A 0·1N KOH solution was used to neutralize the glass-distilled water used in the measurement of the response of *V. cholerae* to different cation combinations and concentrations. The formulation of suspending fluid in each experiment is described in the results section. All survival experiments were initiated by adding 1·0 ml of inoculating suspension to 500 ml suspending fluid. An analysis of the initial cell concentrations achieved by this technique shows an arithmetic mean of 5·2 $\log_{10}$ colony-forming units (c.f.u.)/ml with a standard deviation of 0·2. The bottles used for the study were made of borosilicate glass and had a total volume of 640 ml. These bottles were stored in the dark at room temperature (about 25 °C).

Counting procedure

A volume (0·1 ml) of bacterial suspension or $\log_{10}$ dilutions of this were spread on a 9 cm agar plate of trypticase soy agar (BBL). The diluent used was 1·0 % (w/v) NaCl at pH 8·0. Bacterial counts were derived from counts of individual colonies and expressed as c.f.u./ml. The dilution chosen for the count taken was that which gave between 30 and 300 colonies per plate. Each count was carried out in duplicate and an arithmetic mean calculated.

Sampling timetable

Most of the experiments used a sampling timetable with doubling time intervals. Counts were made at the time of inoculation and as close to the timetable of 1, 2, 4, 8, 16, 32 and 64 days as possible. The counting of any one suspension was discontinued after failure to recover *V. cholerae* on two consecutive samplings.

Composition of minimal medium used to determine the requirement of V. cholerae *for Na^+ for growth*

The vibrios were grown in modified M9 minimal medium mixed with equal volumes of mixtures of NaCl and KCl solutions. The minimal medium used is described by Clowes & Hayes (1968), modified by substituting potassium salts for all the sodium salts. The composition of this medium was 10 ml 20 % glucose, 5 ml 2·46 % $MgSO_4$, 5 ml 0·147 % $CaCl_2$, 180 ml distilled water, 50 ml M9 solution. The ingredients for 1 litre M9 solution were: K_2HPO_4, 76·3 g; KH_2PO_4, 30 g; KCl, 5 g; NH_4Cl, 10 g. The NaCl/KCl mixtures used were prepared to give total concentrations of NaCl in the media of 0 % 0·0005 %, 0·005 %, 0·05 % and 0·5 %, while keeping osmolarity constant with the use of KCl. Analar grade reagents were used throughout.

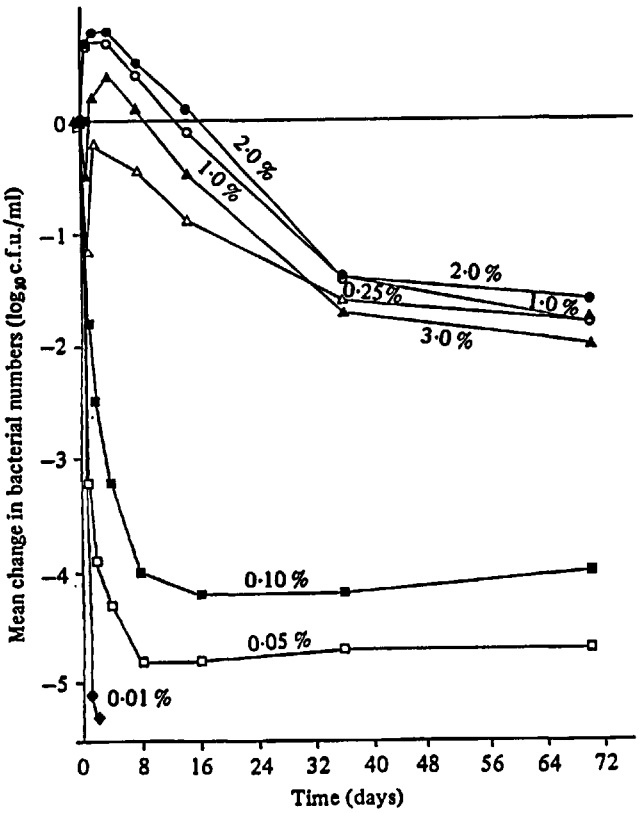

Fig. 1. Response of *V. cholerae* to different sea salt concentrations at 25 °C: 0·01 % (◆), 0·05 % (□), 0·10 % (■), 0·25 % (△), 1·0 % (○), 2·0 % (●), 3·0 % (▲). Each line represents the arithmetic mean of the total change in numbers ($\log_{10}$ c.f.u./ml) from the start of the experiment of six strains, at each salt concentration.

Composition of the simple salt solution used to investigate the interstrain variation in the response of V. cholerae *to favourable conditions*

The composition of the suspension fluid was: $(NH_4)_2SO_4$ 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 osmolarity of this solution was about 254 mOsm and the pH 8·0.

RESULTS

Response of V. cholerae *to different sea salt concentrations*

The six strains of *V. cholerae* 01 (CT^+) were each suspended in nine different concentrations of pure natural sea salt (Maldons) containing no additives, and stored in the dark at room temperature (about 25 °C). Bacterial numbers were monitored for 70 days and the results are described in Fig. 1. The optimal salt concentration for survival was 2·0 %, though all solutions in the range 0·25–3·0 % gave good support to the vibrios. The lowest salt concentration consistent with the stable survival of *V. cholerae* was 0·05 %, and the lowest concentration allowing survival beyond 24 h was 0·01 %. None of the six strains tested survived for 24 h in the 0·001 % and 0·005 % solutions. At salt concentrations of 0·05 % and above,

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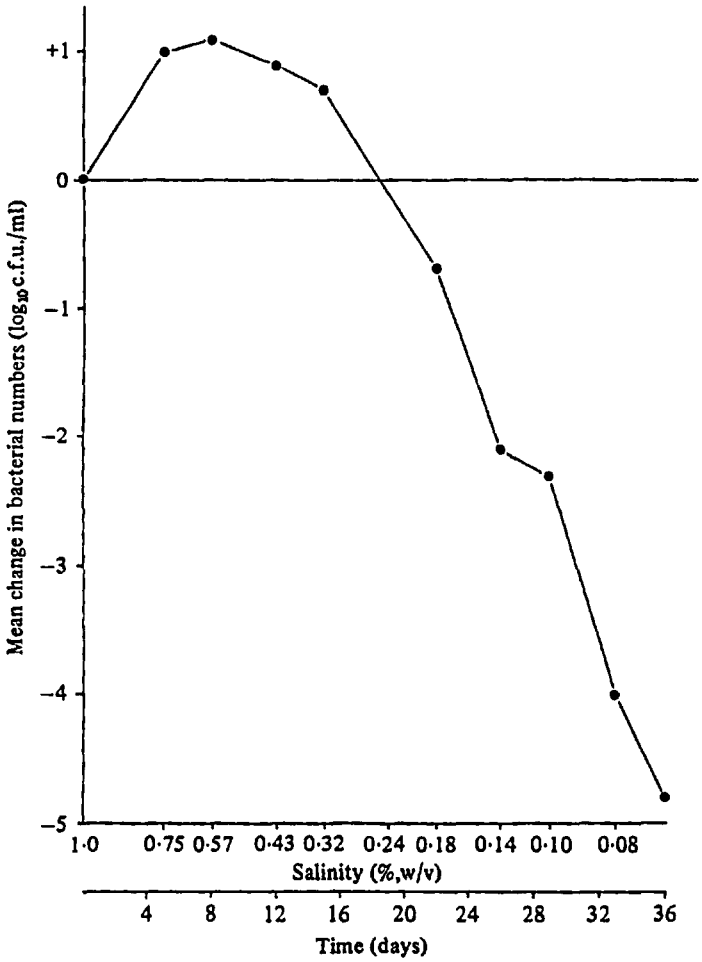

Fig. 2. The adaptation of *V. cholerae* to low salinities at 25 °C. The line represents the arithmetic mean of the total change in numbers (log_{10} c.f.u./ml) from the start of the experiment of six strains.

V. cholerae appear to be able to enter a semi-dormant state in which they cease to require measurable levels of nutrients.

The stabilization of bacterial numbers at low salinities in the latter stages of the experiment indicates that either *V. cholerae* can adapt to low salinities or that there are sub-strains of *V. cholerae* with higher resistance to low salinities than the parent strains. Fig. 2 shows the decline of cells given time to adapt to low salinities. The six strains of *V. cholerae* 01 (CT^+) were each suspended in a 1 % (w/v) solution of sea salt. The salinity of each solution was then gradually decreased by the successive addition of sterile distilled water. Bacterial numbers were monitored for as long as the organisms could be recovered. The 33-day count in this experiment was made from water with a salinity of 0·10 % (counts being made immediately before each dilution). The mean decline in bacterial numbers at this point was 4·0 log_{10} c.f.u./ml. In contrast to this, the mean decline in bacterial numbers after 33 days exposure to a salinity of 0·10 % (Fig. 1) was 4·2 log_{10} c.f.u./ml. The difference between these means is not statistically significant ($P > 0·05$).

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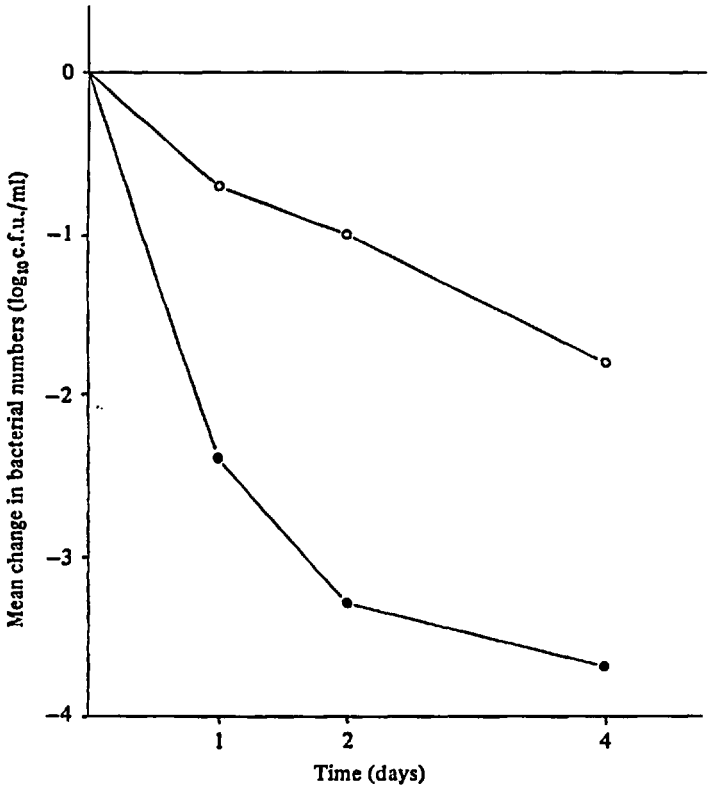

Fig. 3. Intra-strain variation in the response of *V. cholerae* in 0·10% (w/v) sea salt at 25 °C. ○, pre-stressed cells; ●, non pre-stressed cells. Each line represents the arithmetic mean of the total change in numbers (log_{10} c.f.u./ml) from the start of the experiment of three strains.

The stabilization of bacterial numbers at low salinities (Fig. 1) and the inability of *V. cholerae* 01 (CT^+) to adapt to low salinities (Fig. 2) suggest that there are sub-strains of the vibrio with increased resistance to low salinity stress. Fig. 3 shows that resistant sub-strains do exist. Three strains of *V. cholerae* 01 (CT^+) were selected from those that had survived for 8 days in 0·10% sea salt. The bacteria were taken from plates of the day-8-counts, and then resuspended in fresh bottles of 0·10% sea salt. At the same time representatives of the parent strains were removed from the culture collection and suspended in identical bottles of 0·10% sea salt. The pre-stressed strains of *V. cholerae* were seen to possess an increased resistance to stress over the parent strains (Fig. 3).

In addition to the intra-strain variation in stress response demonstrated in Fig. 3, a certain amount of inter-strain variation was also expected. Fig. 4(*a*, *b*) shows the inter-strain variation in response to low salinity among the 59 strains of *V. cholerae* described in Table 1. Each strain was suspended in a solution of 0·05% sea salt (about 19 mOsm) and numbers were monitored over a 73-day period. There was very considerable inter-strain variation; 5 of the 59 strains tested failed to survive even 24 h whereas two strains did not show any decline in numbers after 73 days. Half of the strains (30/59) survived for the 73 days of the experiment. An analysis of the data by Wilcoxons rank test shows that the possession of

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Table 1. *Characteristics of* 59 *strains of* V. cholerae *exposed to* 0·05 % *sea salt*

Serogroup and source	Country of origin			Total no. of strains
	Bangladesh	Tanzania	Australia	
Clinical isolate 01	17	19	0	36
Water isolate 01	3	9	1	13
Water isolate non-01	7	2	0	9
Total strains	27	30	1	58*

* Only 58 strains of *V. cholerae* are described above, the 59th strain included in the experiment was a laboratory reference strain.

resistance to low salinity was not associated with the serogroup of the organism (01 and non-01 serogroups), the medium from which it was isolated (clinical cholera and the environment), or its country of origin (Tanzania and Bangladesh) ($P > 0{\cdot}05$).

Table 2 describes the inter-strain variation of a number of strains of *V. cholerae* under more favourable conditions. Twenty strains of *V. cholerae* were each suspended in a simple salt solution which was stored in the dark at room temperature (about 25 °C) for 40 months. The vibrios were then counted. The counts in Table 2 show a marked degree of uniformity. Fifteen of the 20 counts made at 40 months were in the range 3·2–3·8 $\log_{10}$ c.f.u./ml. This is in marked contrast to the degree of variation in response to a salinity of 0·05 %. The degree of inter-strain variation in stress response therefore appears to be a function of the intensity of the stress. In low stress environments such as those found in this experiment, the degree of variation is small. As stress increases, however, so does inter-strain variation in stress response.

The mean counts of the three groups of strains listed in Table 2 (T/C/01, T/W/01, B/W/non-01) were analysed by the *t* test for means of independent groups. No significant differences between the means were found ($P > 0{\cdot}05$). This result supports that from the previous experiment. Together they fail to show an association between the origin or the serogroup of strains of *V. cholerae* and their response to either favourable or unfavourable conditions.

The results in Table 2 also show that *V. cholerae* can survive for 3 years and more in a simple salt solution without any nutrients and at a warm temperature. The ability to do this is not confined to a small proportion of strains. Of the 20 strains originally put into solution, 18 were still viable at 40 months.

Response of V. cholerae *to different temperatures and salinities*

Fig. 5 describes the survival of *V. cholerae* 01 (CT^+) at three different salinities and at two temperatures. Each of the six strains of *V. cholerae* were suspended in a duplicate series of sea salt solutions of 0·01 %, 0·10 % and 1·0 %. One series of solutions was stored in the dark at room temperature (about 25 °C) and one series was stored in the dark at 4 °C. Results show that temperature and salinity interact in determining the survival of *V. cholerae*. Fig. 5 shows that the vibrios survive longer at 25 °C than at 4 °C, at all salinities. However, the longer survival at 25 °C at 0·01 % salinity was due to one strain only, and five of the six strains tested survived longer at this salinity, at 4 °C. At higher salinities the reverse occurred

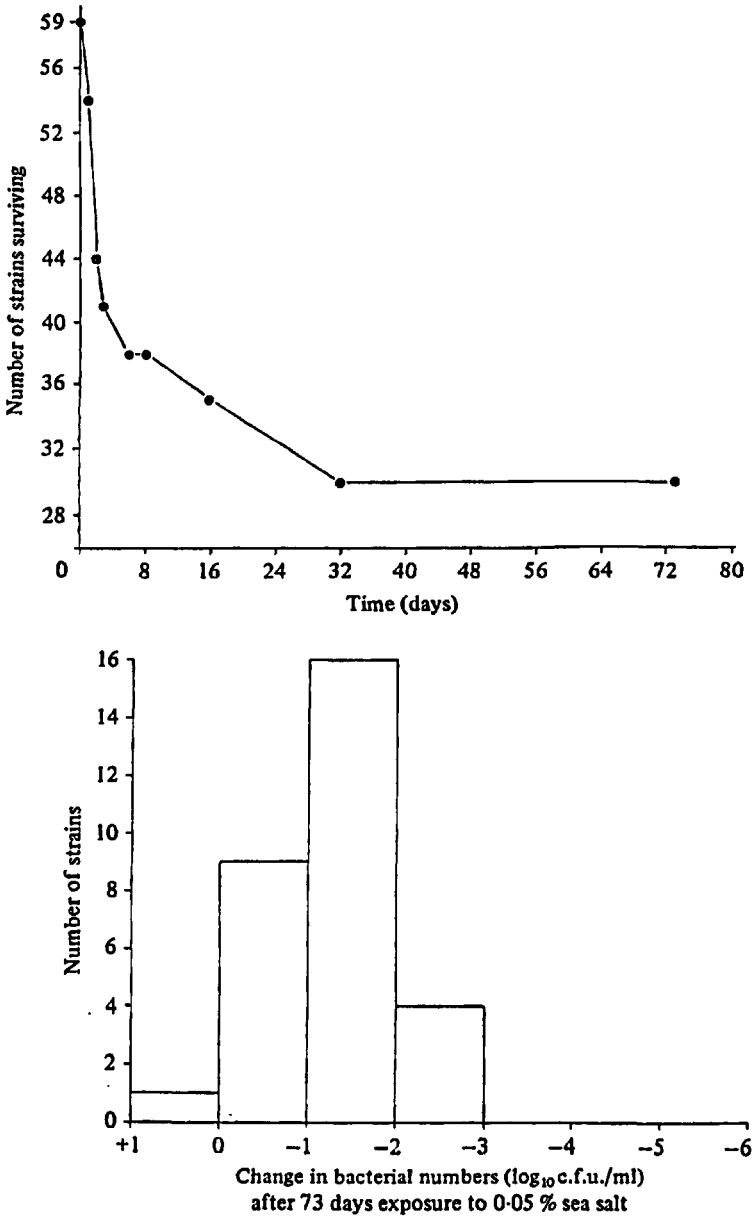

Fig. 4(*a*) Response of 59 strains of *V. cholerae* to suspension in 0·05 % sea salt at 25 °C. (*b*) Change in bacterial numbers of 30 strains of *V. cholerae* after surviving 73 days exposure to 0·05 % sea salt at 25 °C.

and the vibrios survived longer at room temperature. The vibrios could not survive beyond 45 days at 4 °C at any of the three salinities tested.

Fig. 6 describes the survival of the six strains of *V. cholerae* at salinity of 1 % (w/v) at a temperature of 4 °C and in the presence (1 %, w/v, peptone) and absence of nutrients. Results show that *V. cholerae* cannot persist at such a low temperature even if nutrients are present.

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Table 2. *Response of* 20 *strains of* V. cholerae *to* 40 *months suspension in a simple salt solution at* 25 °*C*

Characteristics of strains examined*				Count (log_{10} c.f.u./ml) after 40 months§
Collection no.	Country of origin†	Source of isolate‡	Serogroup	
10	T	C	01	3·4
11	T	C	01	3·4
13	T	C	01	3·4
15	T	C	01	3·2
102	T	C	01	0
103	T	C	01	3·3
104	T	C	01	0
106	T	C	01	3·6
109	T	C	01	2·2
110	T	C	01	3·2
112	T	C	01	3·6
1	T	W	01	3·6
2	T	W	01	3·6
3	T	W	01	3·7
8	T	W	01	2·6
9	T	W	01	3·6
51	B	W	Non-01	2·2
54	B	W	Non-01	3·2
56	B	W	Non-01	3·5
57	B	W	Non-01	3·8

* All clinical isolates examined in this experiment were of the El Tor biotype.
† Country of isolation: T = Tanzania, B = Bangladesh.
‡ Source of isolate: C = clinical cholera, W = water.
§ The estimated initial concentration was 5·2 log_{10} c.f.u./ml.

Response of V. cholerae *to different pH values*

Fig. 7 describes the mean response of the six strains of *V. cholerae* 01 (CT^+) to different pH values while in solutions of favourable osmolarity (340 mOsm, about the same osmolarity as 1·0, w/v, NaCl). Each of the six strains was suspended in di-sodium tetraborate/potassium di-hydrogen phosphate buffer solutions at pH values of 6·0, 6·5, 7·0, 7·5, 8·0, 8·5 and 9·0 and bacterial numbers were monitored over a 64-day period. Survival was successful over the pH range 7·0–8·5 with the optimum being 8·0. pH values 0·5 units either side of this range gave only limited support to the vibrios.

Fig. 8 describes the mean response of the same six strains of *V. cholerae* 01 (CT^+) to the same pH values but at an osmolarity of 34 mOsm (about the same osmolarity as 0·1 %, w/v, NaCl). The response at low osmolarity is similar to that at favourable osmolarity though the pH range for successful survival is shifted 0·5 of a unit higher to 7·5–> 9·0.

Response of V. cholerae *to different cation combinations and concentrations*

Fig. 9 describes the comparative survival of *V. cholerae* 01 (CT^+) in solutions of sea salt and NaCl. *V. cholerae* are shown to survive longer in solutions of sea salt

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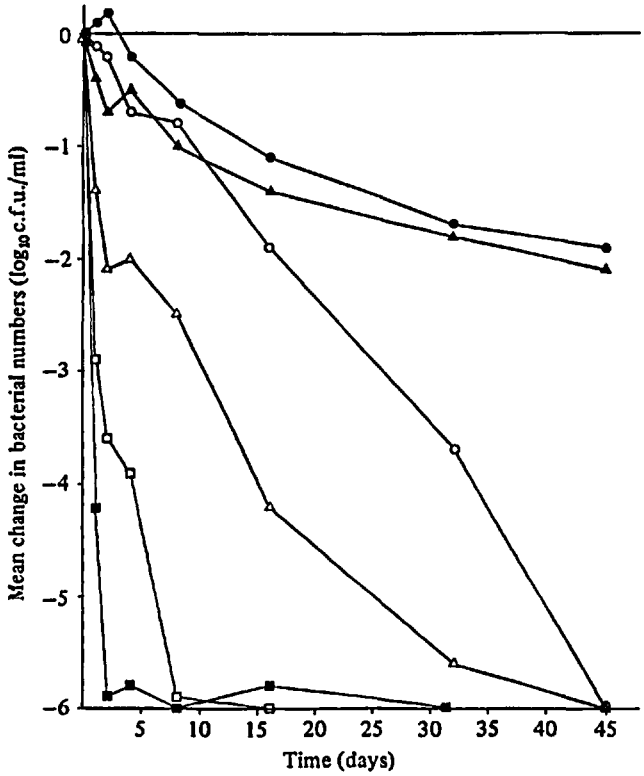

Fig. 5. Response of *V. cholerae* to different temperatures and salinities: 0·01 %, 25 °C (■); 0·10 %, 25 °C (▲); 1·0 %, 25 °C (●); 0·01 %, 4 °C (□); 0·10 %, 4 °C (△); 1·0 %, 4 °C (○). Each line represents the arithmetic mean of the total change in numbers (log_{10} c.f.u./ml) from the start of the experiment of six strains, in each of the conditions.

than in solutions containing equivalent concentrations of NaCl. This is not due purely to differences in osmolarity because the increased survival can be seen at both ends of the concentration spectrum. The increase may therefore be caused by favourable interactions between the cell and the minor sea salt ions. Fig. 10 describes the survival of *V. cholerae* 01 (CT^+) in a range of solutions representing the minor sea water cations. Six strains of *V. cholerae* were each suspended in solutions of NaCl, KCl, $MgCl_2$, $CaCl_2$, $SrCl_2$ and four mixtures of equivalent volumes of NaCl + KCl, NaCl + $MgCl_2$, NaCl + $CaCl_2$ and NaCl + $SrCl_2$. All solutions were prepared to an osmolarity of 340 mOsm.

Results show that *V. cholerae* respond favourably to suspension in mixtures of Ca^{2+} with Na^+ and of Mg^{2+} with Na^+ during the first 8 days, although by 64 days numbers were similar to those in NaCl alone. These divalent cations may be responsible for the increased survival of *V. cholerae* in sea salt solutions. Survival was comparatively poor in the Sr^{2+} with Na^+ and in the K^+ with Na^+ mixtures.

A striking result from Fig. 10 is that *V. cholerae* did not survive beyond 4 days in the sodium-free solutions of $SrCl_2$, $CaCl_2$ and $MgCl_2$. Limited survival was seen in the KCl solution but this was confined to only one of the six strains tested. The rapid decline in these solutions was not due to the toxicity of the cations as it was not observed when these solutions were mixed with NaCl. *V. cholerae* 01 (CT^+) appear to have a requirement for sodium.

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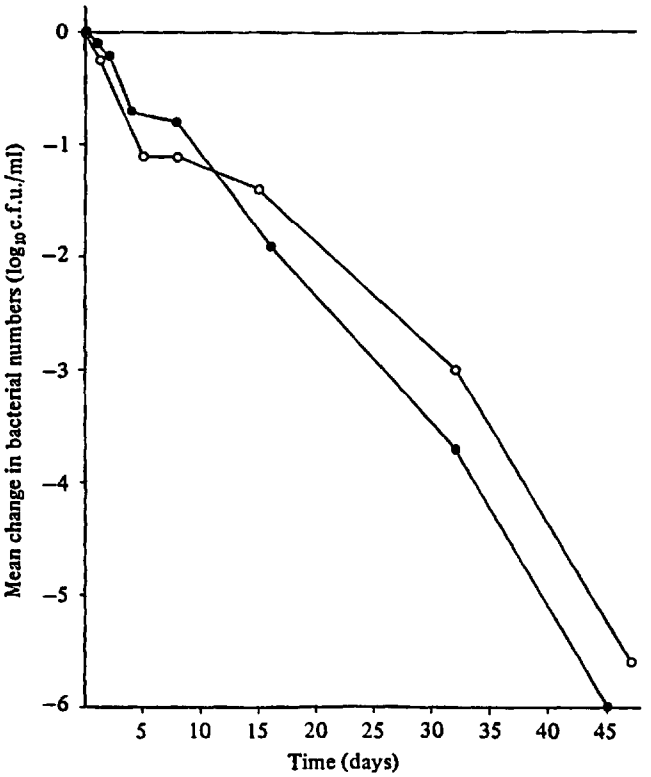

Fig. 6. Response of *V. cholerae* to suspension in 1·0 % (w/v) sea salt in the presence (○) and absence (●) of nutrients at 4 °C. Each line represents the arithmetic mean of the total change in numbers (log_{10} c.f.u./ml) from the start of the experiment of 6 strains, in each of the conditions.

Fig. 11 describes the survival of *V. cholerae* 01 (CT^+) in different concentrations of sodium. Six strains of *V. cholerae* were each suspended in a range of solutions containing different Na^+ concentrations. Osmolarity was maintained at 680 mOsm (about the same osmolarity as 2·0 %, w/v, NaCl) with the use of KCl. Bacterial numbers were monitored for 77 days.

Results show that *V. cholerae* 01 (CT^+) do require Na^+ for survival. The organisms did not survive at all in the absence of Na^+ and only just survived the length of the experiment in the 0·001 % NaCl/KCl solution. Increase in Na^+ concentration beyond this level gave increased support to the vibrio. This result raises the question of whether it is osmolarity or Na^+ concentration that determines the survival of *V. cholerae* in solutions of sea salt (Fig. 1).

Fig. 10 shows that mixed solutions of KCl and NaCl are less able to support the survival of *V. cholerae* than solutions of NaCl. Fig. 9 shows that NaCl is less supportive than sea salt. Survival in NaCl is therefore a better model for the survival of *V. cholerae* in Na^+/K^+ mixtures than survival in sea salt solutions. Fig. 12 describes the survival of *V. cholerae* 01 (CT^+) in a range of NaCl solutions. The protocol followed was identical to that used for the sea salt experiment (Fig. 1).

Figs. 11 and 12 show that sodium concentration only becomes limiting at concentrations several orders of magnitude below that required for survival if the salt is also needed for the provision of osmotic support. For example, 0·001 % NaCl

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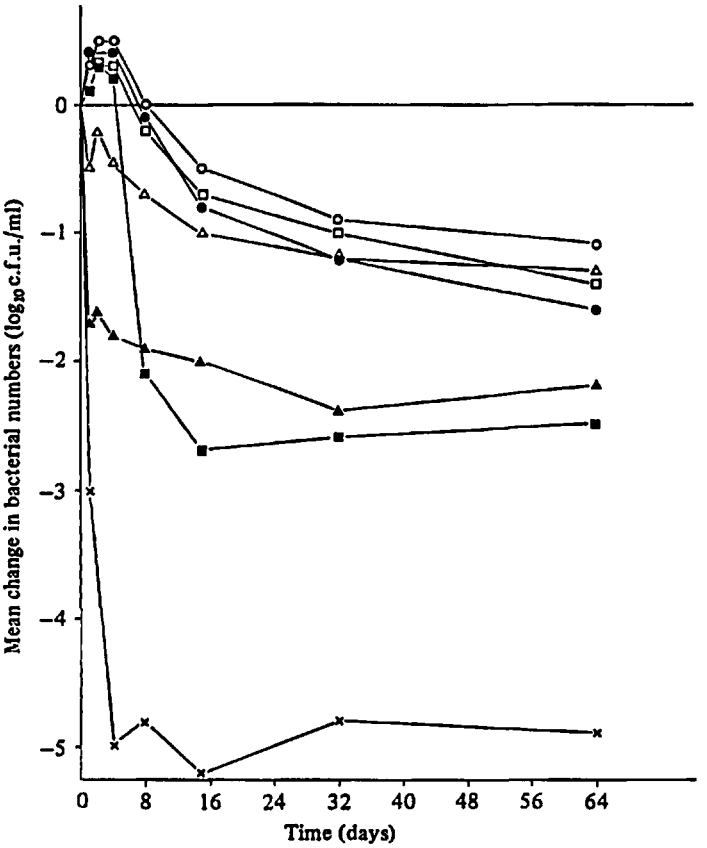

Fig. 7. Response of *V. cholerae* to different pH values at 340 mOsm and 25 °C: (×), 6·5 (▲), 7·0 (△), 7·5 (□), 8·0 (○), 8·5 (●), 9·0 (■). Each line represents the arithmetic mean of the total change in numbers (log_{10} c.f.u./ml) from the start of the experiment of six strains, at each pH value.

can just supply enough sodium for survival whereas a salt concentration 100 times greater than this will not meet the cells' osmotic requirements. Osmolarity therefore plays a major role in the survival of *V. cholerae* 01 (CT^+) and can be assumed to have a greater impact on survival than the effect of low sodium. At low salinities it is osmolarity rather than Na^+ concentration which is the dominant factor influencing survival.

The finding that *V. cholerae* 01 (CT^+) require Na^+ for survival is surprising in view of the current description of *V. cholerae* as organisms that can grow in 1% tryptone without added NaCl (Hugh & Feeley, 1972; Hugh & Sakazaki, 1972). There are three possible explanations for this: the provision of energy may remove the dependence of *V. cholerae* 01 (CT^+) on Na^+ for survival and growth, the provision of energy may remove the dependence on Na^+ for survival but not for growth (and 1% tryptone contains enough Na^+ to support the growth of these organisms), or the provision of energy does not remove the dependence on Na^+ for growth or survival but 1% tryptone contains enough Na^+ for both these

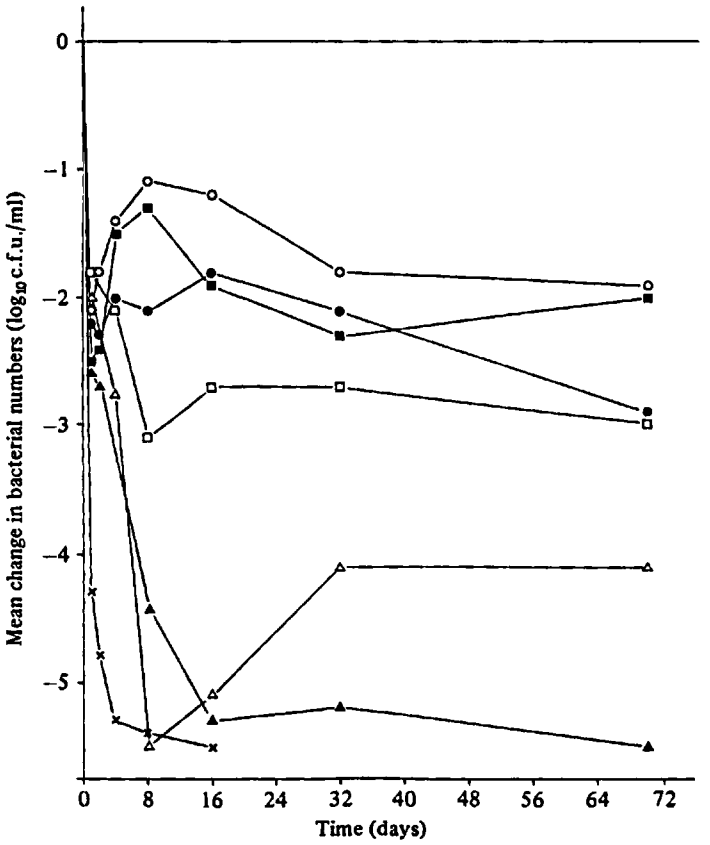

Fig. 8. Response of *V. cholerae* to different pH values at 34 mOsm and 25 °C: 6·0 (×), 6·5 (▲), 7·0 (△), 7·5 (□), 8·0 (○), 8·5 (●), 9·0 (■). Each line represents the arithmetic mean of the total change in numbers (log_{10} c.f.u./ml) from the start of the experiment of six strains, at each pH value.

functions. Fig. 13 describes the response of *V. cholerae* 01 (CT^+) to different sodium concentrations in the presence of nutrients. The six strains of *V. cholerae* were each inoculated into modified M9 minimal medium containing a range of Na^+ concentrations. Counts were made after 5 and 24 h at 37 °C.

Results show that if nutrients are present and Na^+ absent, populations of *V. cholerae* remain static, although the addition of low concentrations of Na^+ will allow the organisms to grow. This supports the second explanation given above – that the provision of energy removes the dependence of *V. cholerae* 01 (CT^+) on Na^+ for survival but not for growth, and the growth of these organisms in 1 % tryptone is supported by the low levels of Na^+ in this medium (Difco Laboratories, 1953).

These results also show that the optimal concentration of Na^+ for growth is comparatively high. After 5 h incubation no growth at all was seen in the absence of sodium whereas the vibrio concentrations in the other bottles had increased by an amount roughly proportional to sodium concentration. After 24 h a small increase in bacterial numbers was seen in the bottles not containing sodium

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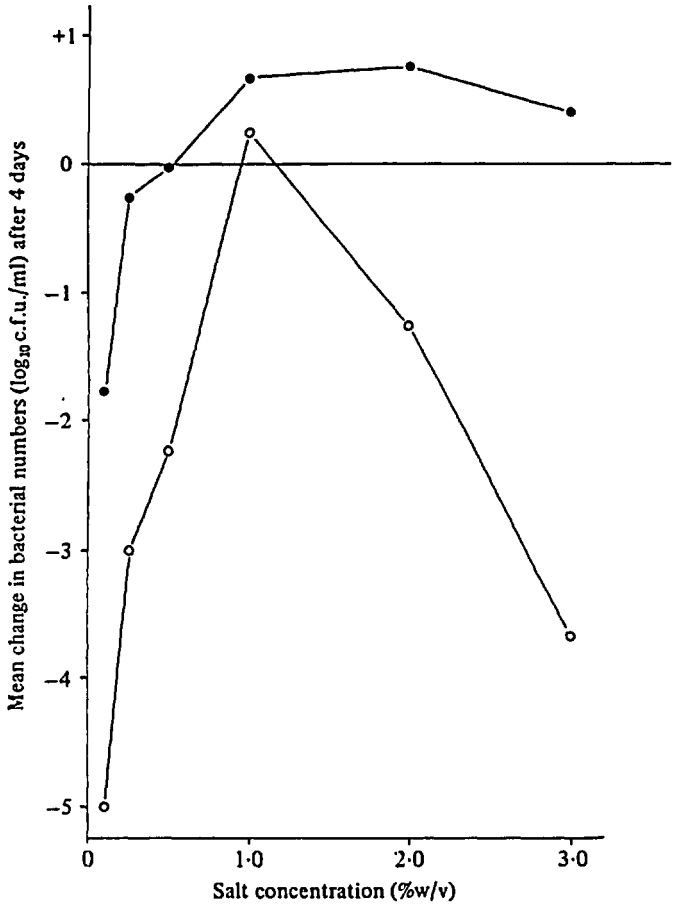

Fig. 9. Comparative survival of *V. cholerae* in solutions of sea salt (●) and NaCl (○): mean response after 4 days at 25 °C. Each line represents the arithmetic mean of the total change in numbers (log_{10} c.f.u./ml) from the start of the experiment of six strains.

whereas bacterial numbers were inversely proportional to Na^+ concentration in the other solutions. This effect is presumably due to a temporal displacement of the growth curves, with low NaCl concentrations causing a slower growth rate or a longer lag phase or both.

DISCUSSION

If *V. cholerae* 01 (CT^+) are to survive within the environment they must be able to compete successfully with other environmental organisms for nutrients and be able to survive when nutrients are not available. To compete successfully the organisms must be able to utilize nutrients before their competitors are able to do so. Fig. 13 shows that the speed of nutrient utilization is dependent on Na^+ concentration and is fastest at the highest Na^+ concentration tested. Nutrient utilization is most rapid at Na^+ concentrations given by salinities greater than 0·5 %. The relative growth rate of bacteria is not only dependent on physico-chemical factors such as salinity but also on nutrient concentration (Jannasch, 1968). *V. cholerae* 01 (CT^+) grow comparatively rapidly on the high levels of nutrients found

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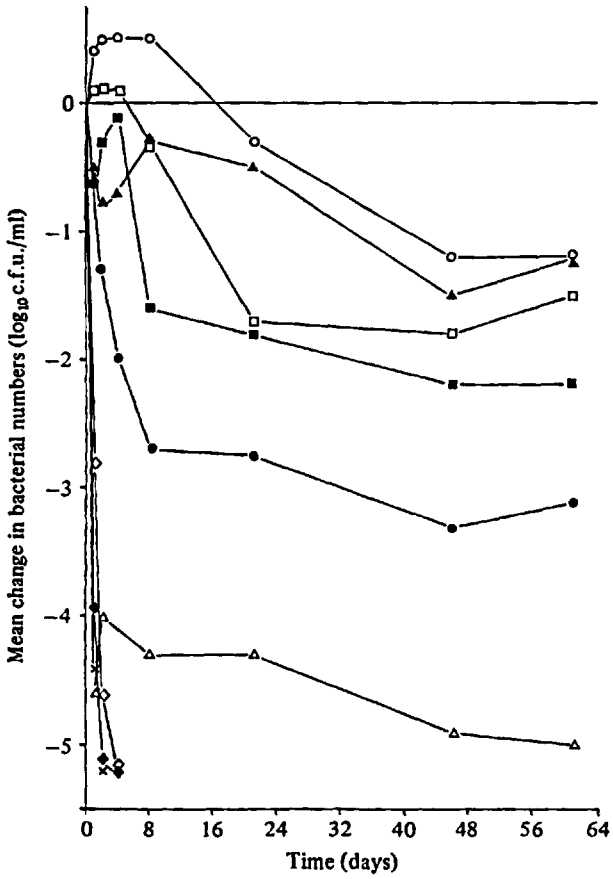

Fig. 10. Response of *V. cholerae* to suspension in a number of different salt solutions at 340 mOsm and 25 °C: $SrCl_2$ (×), $CaCl_2$ (◆), $MgCl_2$ (◇), KCl (△), NaCl (▲), $SrCl_2$+NaCl (●), $CaCl_2$+NaCl (□), $MgCl_2$+NaCl (○), KCl+NaCl (■). Each line represents the arithmetic mean of the total change in numbers (log_{10} c.f.u./ml) from the start of the experiment of six strains, in each of the salt solutions.

in conventional laboratory media. It therefore appears that *V. cholerae* 01 (CT^+) are adapted to compete at high nutrient levels and at salinities of > 0·5 %. Many high-nutrient habitats exist within estuaries. An example of such a habitat, containing nutrients available to *V. cholerae*, is the surface of a piece of chitin.

In contrast to this result Singleton *et al.* (1982*a*) found that, whereas *V. cholerae* 01 (CT^+) require a salinity of 2·5 % for optimal growth at low nutrient concentrations, no association between growth and salinity was seen at high nutrient concentrations such as those used in our study. This apparent discrepancy may be explained by the fact that Singleton *et al.* only measured growth in the salinity range 1·5–4·5 % whereas in our study the Na^+ concentrations corresponded to salinities of 0–0·5 %. The effect of salinity on the growth of *V. cholerae* may be greater at low salt concentrations.

The ability of *V. cholerae* to grow in minimal media has been recognized for some time (Pollitzer, 1959). We have found M9 medium to support the rapid growth of *V. cholerae* 01 and non-01 and *V. cholerae* (CT^+) and (CT^-) for a number of different

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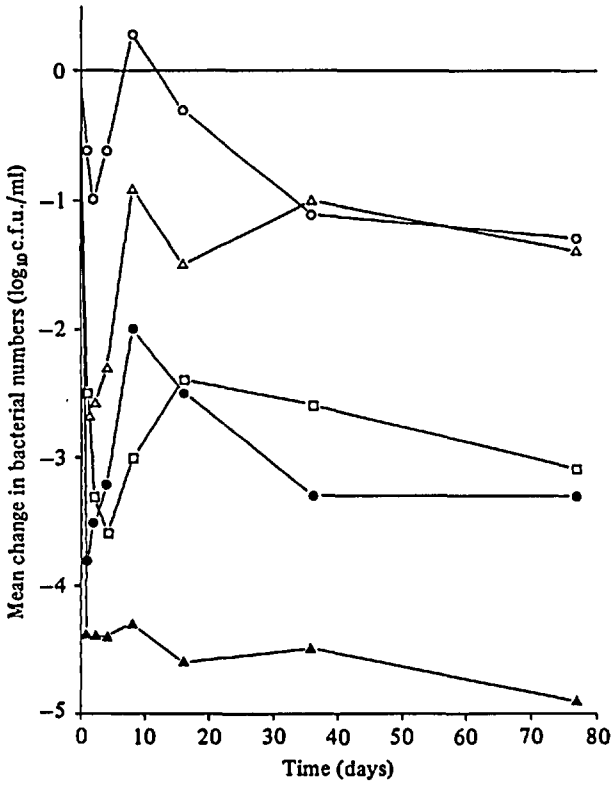

Fig. 11. Response of *V. cholerae* to suspension at 25 °C in solutions with different sodium concentrations but with osmolarity maintained at 680 mOsm with KCl: 0·001 % NaCl (▲), 0·01 % NaCl (●), 0·10 % NaCl (□), 1·0 % NaCl (△), 2·0 % NaCl (○). Each line represents the arithmetic mean of the total change in numbers (log_{10} c.f.u./ml) from the start of the experiment of six strains, in each of the salt solutions.

countries (unpublished data). This medium does not contain amino acids. Singleton *et al.* (1982*b*) concluded that since tryptone alone supported growth of *V. cholerae* and glucose had to be supplemented with yeast extract to support growth, *V. cholerae* require one or more vitamins or other factors for growth in addition to Na^+. The growth of *V. cholerae* in M9 medium shows that the organism can obtain its nitrogen from inorganic sources (in this case NH_4Cl).

The ability of an organism to exist within the environment also depends on its ability to survive when nutrients become scarce. If the survival strategy of *V. cholerae* 01 (CT^+) is to utilize discrete particles of food like chitin, or if they survive by floating in the planktonic phase, they must be able to withstand periods of low nutrient availability. The results of this study show that, if an estuarine salinity, a pH of 7·0–8·5 and a warm temperature are provided, the organisms can survive for long periods without nutrients. The organisms appear to enter a dormant state when deprived of nutrients and the mortality rate while in this state is very low. Baker, Singleton & Hood (1983) have recently shown that *V. cholerae* exhibit a change in morphology to small coccoid forms during periods of starvation.

The requirement of the vibrio for comparatively high salinities also resolves the conflict between the results of most previous studies on cholera survival which show

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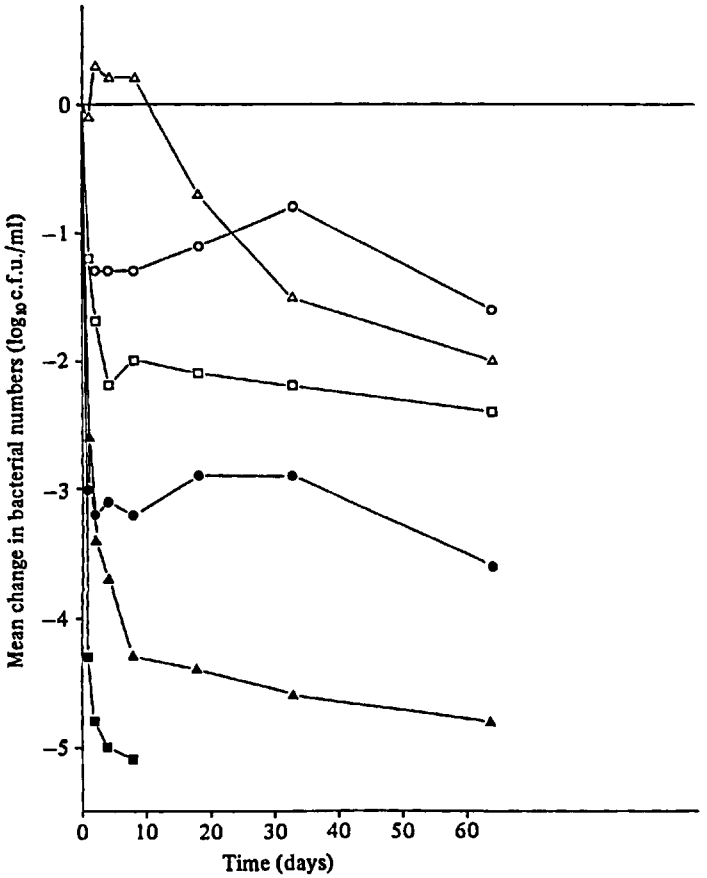

Fig. 12. Response of *V. cholerae* to different NaCl concentrations at 25 °C: 0·10% (■), 0·25% (●), 0·50% (□), 1·0% (△), 2·0% (○), 3·0% (▲). Each line represents the arithmetic mean of the total change in numbers ($\log_{10}$ c.f.u./ml) from the start of the experiment of six strains, in each of the salt solutions.

the organisms to be labile (Felsenfeld, 1974; Pollitzer, 1959) and the environmental isolation of the organisms in conditions which suggest that they are normal inhabitants of the environment (Blake *et al.* 1980; Rogers *et al.* 1980). Most previous studies were designed to evaluate the role of drinking water in the transmission of cholera and were consequently carried out in water of low salinity. Fig. 1 shows that the survival of the organisms is limited in drinking water whereas in estuarine waters of higher salinity, from which the environmental isolations have been made, survival is protracted. It is possible, however, that the requirement for estuarine salinities may be modified if the *V. cholerae* are in close association with certain aquatic microhabitats, such as zooplankton. A similar modification of salinity dependence has been recently proposed for *V. parahaemolyticus* (Sarker *et al.* 1983).

The high level of inter-strain variation in the response of *V. cholerae* to water of low salinity (Fig. 4*a*, *b*) emphasizes the risk of carrying out survival studies on *V. cholerae* with only one or two strains of the species. A number of such studies have appeared in the literature. They should be interpreted with caution.

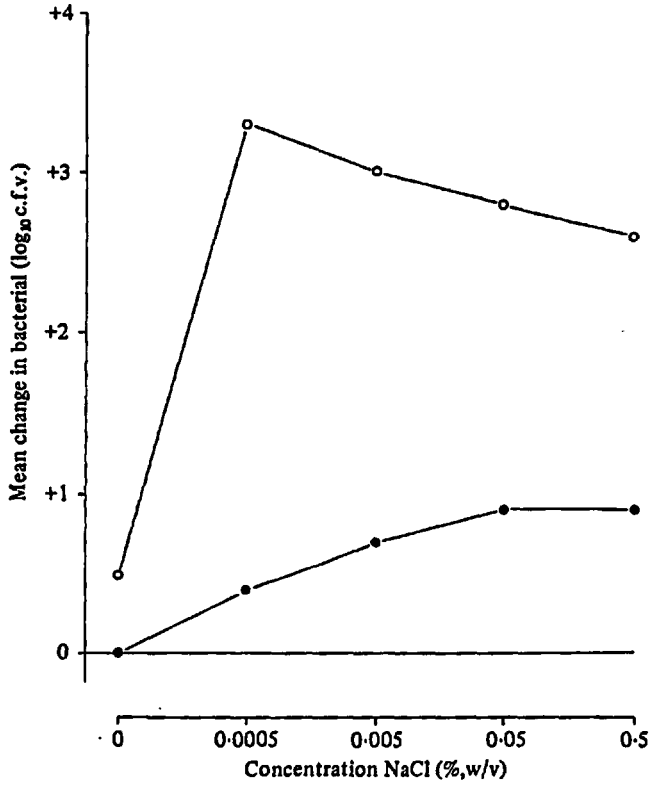

Figure 13. Response of *V. cholerae* to different sodium concentrations in modified M9 minimal medium after 5 h (●) and 24 h (○) at 37 °C. Each line represents the arithmetic mean of the total change in numbers (log_{10} c.f.u./ml) from the start of the experiment of six strains.

Two other aspects of the results support the hypothesis that *V. cholerae* 01 (CT^+) can survive in the environment. The demonstration that *V. cholerae* 01 (CT^+) and *V. cholerae* non-01 (CT^-) have the same survival characteristics shows that the carriage of the tox gene and the 01 antigen do not make the organism more susceptible to stress than the non-01 (CT^-) strains which are known to be residents of certain aquatic environments. The requirement of Na^+ for growth (Fig. 12), also shown by Singleton *et al.* (1982*b*), further supports the hypothesis because this is a characteristic of marine organisms (MacLeod, 1965), though some terrestrial organisms do have a similar requirement. Fig. 8 emphasizes the requirement for Na^+ by showing the death of *V. cholerae* in its absence when nutrients are also absent.

Laboratory evidence suggests therefore that *V. cholerae* 01 (CT^+) are part of the normal flora of some aquatic environments. The chief factors determining the distribution of the organism within the aquatic environment are salinity and temperature. Salinity will not only determine the length of survival in low or zero nutrient waters but also the ability of the organism to compete for nutrients when they are available. The salinity requirements of the organism will restrict its distribution to saline water. The inability to adapt to low salinities will prevent

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the organism from colonizing waters of potable salinity. *V. cholerae* have a requirement for Na^+ separate from their requirement for osmotic support provided by sea salt, though this requirement will not be limiting in natural waters.

The demonstration that *V. cholerae* require Na^+ for growth questions the validity of describing *V. cholerae* as organisms that can grow in 1% tryptone without added NaCl. The results of this test will be dependent on the level of Na^+ in the tryptone and may be misleading if a low sodium tryptone is used.

The distribution of cholera vibrios in the environment will also be governed by water temperature. *V. cholerae* are unable to grow and utilize nutrients at temperatures below 10–12 °C (Pollitzer, 1959). Results from this study have shown that the organisms die at a temperature of 4 °C regardless of salinity and nutrient levels. This will restrict the habitat of the organisms to those areas where the winter water temperature does not fall to such low values. The winter water temperatures of Kent and Chesapeake Bay remain below 10–12 °C for extended periods and may fall to 4 °C. Kaper *et al.* (1979, 1981), Colwell *et al.* (1981) and Lee *et al.* (1982) have isolated *V. cholerae* non-01 and 01 (CT^-) from these areas, mostly during the summer, and have concluded that the organisms are part of the normal flora of these areas. If *V. cholerae* non-01 and 01 (CT^-) have a similar response to low temperature to *V. cholerae* 01 (CT^+), they may be unable to persist through the winter at these latitudes. They may, however, be seasonally re-introduced to these waters by birds. Ducks and other birds have been shown to carry *V. cholerae* (Lee *et al.* 1982; Bisgaard & Kristensen, 1975; Bisgaard, Sakazaki & Shimada, 1978). Alternatively, the vibrios may retreat to specialized microhabitats, not reproduced in our experiments, where they are able to survive despite low temperatures.

The control of cholera is based on the understanding of the epidemiology of the disease, and this in turn is based on the understanding of the ecology of *V. cholerae* 01 (CT^+). The maintenance of cholera through the non-cholera season in endemic areas may be due to an aquatic reservoir. The data presented here suggest that candidate aquatic reservoirs have salinities of 0·25–3·00%, that they are typically warm, and that their temperatures do not fall to below 5 °C for extended periods. Detailed field studies are required to determine if reservoirs of *V. cholerae* 01 (CT^+) really exist in such aquatic environments in the endemic areas of Asia and Africa.

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