Phage-induced change of toxigenesis in *Vibrio cholerae*

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Summary. A temperate phage coding for constitutive hypertoxigenicity has been constructed in *Vibrio cholerae* strain 569B and used to lysogenise the low-toxin-producing strain MAK 757; 18% of lysogens showed 10–100-fold increase in toxin production. This property was also transmitted at low frequency to second generation lysogens. Thus temperate phage can increase toxin production in a low-toxin-producing strain.

Introduction

Pathogenic strains of *Vibrio cholerae* usually produce moderate levels of toxin *in vitro* whereas environmental strains do not. The possibility for evolution of a toxigenic variant from environmental vibrios by acquisition of a temperate phage therefore poses an intriguing question.

*V. cholerae* strain 569B is unusual in that it is constitutively hypertoxicogenic and carries two defective phages, VcA-1 and VcA-2 (Gerdes and Romig, 1975a). Toxigenesis is a chromosomally determined character in *V. cholerae* (Vasil et al., 1975) and is not affected by curing strain 569B of its defective phages (Gerdes and Romig, 1975b). Generalised transduction of nutritional and antibiotic resistance markers in *V. cholerae* has been reported (Ogg et al., 1981).

This study was performed to determine whether temperate phage can increase toxin production in a low-toxin-producing strain of *V. cholerae*.

Materials and methods

Bacteria and phages

*V. cholerae* strains used were classical biotype strain 569B (serotype Inaba); phage propagating eltor biotype strain MAK757 (serotype Ogawa); immunotyping lysogen strains MAK757(a), MAK757(VcA-1) and MAK757(β) (Siddiqui and Bhattacharyya, 1984); K-class phage indicator strains H218 and MAK757.

Bacteriophages used were lytic typing phages of the eltor and classical sets: temperate K-class phage α and VcA-2, phage β and heteroimmune phage VcA-1 (Siddiqui and Bhattacharyya, 1982, 1983 and 1984).

Details of source, maintenance, assay and construction of lysogens have been given elsewhere (Siddiqui and Bhattacharyya, 1982). The preparation of phage β and its lysogens has been described elsewhere (Siddiqui and Ghosh, 1983; Siddiqui and Bhattacharyya, 1984). The cultures and their derivatives were monitored for serotype and biotype stability and their phages characterised by immunotyping. This was done by spotting phage lysates of the cultures on the three immunotyping lysogens and also testing their sensitivity to the immunotype phages in a similar manner (Siddiqui and Bhattacharyya, 1984).

Toxin production and assay

Flasks (100 ml) containing 30 ml of peptone water (Difco, 2%; pH 7.4; containing NaCl 0.5%) were lightly inoculated from an agar culture and incubated for 18 h at 30°C with shaking. Thiomersal was added to give a final concentration of 0.01% and the pH was adjusted to 6.5 to stabilise toxicity (Bhattacharyya 1973). Growth was monitored spectrophotometrically at 580 nm before centrifugation in the cold at 16,000 g to remove cells. Culture supernates were assayed for cholera toxin by the rabbit skin permeability test (Craig, 1966). Initial screening was at dilutions in saline of 10² and 10³. Lines that gave blueing zones of 6 mm at dilutions of 10² were kept aside for end-point assay between 10² and 10⁻¹ dilutions. A 1–3 mm diameter of blueing zone was considered a negative result. Preparations from strains 569B, MAK757 and immunotyping lysogens were included as controls.

Results

Curing of strain 569B and lysogenisation with phage α

Strain 569B contains two defective phages, VcA-1 and VcA-2 (Gerdes and Romig, 1975a), and was found to be resistant to superinfection by both these phages. VcA-2 is a K-class phage with which lytic E3 phage has co-immunity (Siddiqui and Bhattacharyya, 1982). Phage E3 was, therefore,
used to screen colonies of strain 569B for spontaneously arising sensitive segregants in a crossstreaking assay (Siddiqui and Bhattacharyya, 1983). Curing in these segregants, 569B-1S, was confirmed by demonstrating their sensitivity to phage VcA-2. In separately maintained stock cultures of strain 569B from different laboratories, the frequency of curing was found to vary from 5 to 20%. Curing could not be demonstrated in E3-resistant colonies that had been streaked and retested.

When tested for sensitivity to all the immunotyping phages, it was found that the 569B-1S segregants were cured of K-class phage, but retained VcA-1 immunity. Segregants 569B-1S could subsequently support the growth of K-class phages, including VcA-2. A line of hypertoxigenic strain 569B had now been obtained into which a known phage could be introduced for investigation as a vector of toxigenicity.

Cured segregant 569B-1S was infected with K-class phage a. Progeny clones were then found to have regained E3-resistance and were designated 569B-ISR. No change in serotype or biotype was observed and these clones retained resistance to VcA-1.

Progeny clones 569B-1SR were next screened for phage productivity by plating on indicator strains MAK757 and H218. However, only 28 out of 100 colonies were productive lysogens; the remainder were defective. Only two clones, designated 569B-1SRT, that produced turbid plaques on strain MAK757 were obtained.

**Toxin production in lysogens**

Temperate phage from lysogen 569B-1SRT was plated on strain MAK757 and turbid plaques were subcultured and tested. No change in serotype or biotype of strain MAK757 was observed but these lysogens had become resistant to phage a. They were screened for toxigenicity. Of the 40 tested, seven produced toxin at levels higher than strain MAK757 by at least 10-fold. They were designated strains MAK757H(a). Four of these strains had become hypertoxigenic; they produced toxin titres 100-fold higher than strain MAK757, similar to the titres obtained with strain 569B (table I). Phages from these lysogens were again used for constructing second generation lysogens in strain MAK757. Of the subcultures from 30 plaques obtained from this cross, four had retained hypertoxigenicity at the original levels.

Cross-immunity patterns of the first generation progeny of strain MAK757 were studied to determine whether there was any correlation between

<table>
<thead>
<tr>
<th>Culture*</th>
<th>10²</th>
<th>10³</th>
<th>10⁴</th>
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<tbody>
<tr>
<td><strong>Wild type strains</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>569B</td>
<td>10</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>MAK757</td>
<td>6</td>
<td>2</td>
<td>0</td>
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<tr>
<td><strong>Standard immunotype lysogens</strong></td>
<td></td>
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<td></td>
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<tr>
<td>MAK757(VcA-2)</td>
<td>6</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>MAK757(VcA-1)</td>
<td>7</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>MAK757(β)</td>
<td>6</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>MAK757(α)</td>
<td>6</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td><strong>MAK757 lysogens of phage obtained from strain 569B</strong></td>
<td></td>
<td></td>
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<tr>
<td>MAK757H6(α)</td>
<td>7</td>
<td>5</td>
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<tr>
<td>MAK757H10(α)</td>
<td>6</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>MAK757H102(α)</td>
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<td>6</td>
<td>0</td>
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<td>MAK757H11(α)</td>
<td>10</td>
<td>6</td>
<td>5</td>
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<tr>
<td>MAK757H47(α)</td>
<td>7</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>MAK757H61(α)</td>
<td>8</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>MAK757H130(α)</td>
<td>9</td>
<td>8</td>
<td>7</td>
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*a Phages were derived from strain 569B as described in text. 

+ + 3 mm diameter of blueing zone was considered to be a negative result.

**Table II. Characterisation of phages produced by hypertoxigenic strains MAK757H induced by α phage derived from strain 569B**

<table>
<thead>
<tr>
<th>Immunotype strains</th>
<th>Sensitivity to phages from hypertoxigenic lysogens</th>
<th>Sensitivity to immunotype phages</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>MAK757H(a)</strong></td>
<td><strong>α</strong> VcA-1 <strong>β</strong></td>
</tr>
<tr>
<td></td>
<td>47 10 61 11 6 102 130</td>
<td>+ + + + + + + + + +</td>
</tr>
</tbody>
</table>

* + = sensitive; − = resistant. 

immunity profile and toxigenicity of lysogens. We investigated 142 subcultures, of which only the data for the seven lysogens that were hypertoxigenic are given in table II. Similar patterns were obtained with other lysogens. The sensitivity of strains
MAK757H(α) to the immunotyping phages and the activity of their phages on immunotype lysogens were determined. Three classes of immunotype were found in these strains (table II); there was no correlation with the degree of toxigenicity gained.

Discussion

Toxigenic strains of V. cholerae can give rise to both hypo- and hypertoxicogenic variants (Mekalanos and Murphy, 1979a). These are thought to differ in toxigen synthesis at a regulatory level (Mekalanos et al., 1979b). Strain 569B is constitutively hypertoxicogenic and produces 100 times more toxin in vitro than does strain MAK757. Our aim was to transmit hypertoxicogenicity from strain 569B to strain MAK757 by use of a temperate phage as vector. However, strain 569B is immune to superinfection by K-class and VcA-1 temperate phages because it is a double lysogen for these phages. In strain 569B, both phages are defective and non-infective (Gerdes and Romig, 1975a); therefore, spontaneously cured phage-sensitive variants could be expected to arise and to accumulate in the population because they would escape reinfection. Consistent with this view, colonies of strain 569B that were cured of VcA-2 occurred at high frequency in stock cultures, but not in subcultures, derived from single colonies.

Cured segregants such as 569B-1S still retained defective VcA-1 but could be used to construct lysogens with K-class phage α. However, most of these lysogens, e.g., 569B-1SR, were still defective for both α and VcA-1, i.e., they were immune to superinfection but were not phage productive. This indicated that the original defect probably involved the K-class attachment region in the strain 569B chromosome rather than the VcA-2 genome. A minority of lysogens, e.g., 569B-1SRT, were productive of temperate phages. These phages could then be used to infect strain MAK757 allowing analysis of changes in toxigenicity in this host. The derived α phages derived from strain 569B caused an increase in toxigenesis in 18% of MAK757H(α) progeny. Analysis of second generation lysogens of phage from one of these hypertoxicogenic lines in strain MAK757, MAK757H130(α), indicated that the phage carried information encoding hypertoxicogenicity to a recipient strain. Neither of the wild type phages were able to alter toxin production in their parental lysogens.

Amongst the hypertoxicogenic lysogens, two classes were observed; three lysogens showed a 10-fold increase to moderate levels of toxin production, and four showed a 100-fold increase, equivalent to the level produced by strain 569B. Although toxin production in V. cholerae is influenced by several factors the most likely explanation of these results is alteration of expression of a regulatory gene. Quantitative differences in toxin production following heterologous conjugation between strains 569B and MAK757 have been reported by Miller and Mekalanos (1985). They have shown that the resulting differences in toxin production are due to differences in the ability of the toxR gene from different strains to activate the ctx gene(s).

Most V. cholerae are lysogenic (Takeya et al., 1965; Gerdes and Romig, 1975b), with either productive or defective phage(s) and the involvement of temperate vibrio phages in transduction (Ogg et al., 1981), deletion (Mekalanos et al., 1982), conjugation (Johnson and Romig, 1979) and mutation (Johnson et al., 1981) is important. In nature, the role of temperate phages in converting environmental non-pathogenic strains to pathogenic strains by constant interaction with toxigenic vibrios is also likely to be significant.

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