Novel Transmissible Factors in a Non-O1 Vibrio cholerae and a Vibrio sp.

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Transmissible factors encoding production of lacunae (L factors) were demonstrated in a non-O1 Vibrio cholerae and a Vibrio sp. of recent environmental origin. Lacunae were produced in lawns of non-O1 V. cholerae indicator strains under the same assay conditions as those where lacunae were produced by the well characterized P fertility plasmid of V. cholerae O1 and the V fertility factor found in a non-cholera vibrio strain. The origin of the lacunae produced by strains harbouring the V and L factors was examined. No vibriocin or phage activity was found in culture supernates or in lacunae produced by the strains, suggesting that, as in the case of the P plasmid, the lacunae probably represent sites of active mating. Unlike the P plasmid, neither the Vn or L factor could be detected or isolated by conventional plasmid techniques.

INTRODUCTION

Non-O1 Vibrio cholerae are found in brackish waters and estuaries (Colwell et al., 1977, 1980; Kaper et al., 1979) and are being increasingly recognized as agents of diarrhoeal illness. Some pathogenic non-O1 strains produce cholera toxin (Blake et al., 1980; Craig et al., 1981; Kaper et al., 1981) whereas others produce illness by less clearly defined mechanisms (Spira & Daniel, 1980). The terms V. cholerae O1 and non-O1 are used to distinguish between the O-group 1 serovar responsible for epidemic and endemic cholera and V. cholerae strains which belong to other serovars. Non-O1 V. cholerae were formerly included under the non-specific designation non-cholera vibrios (NCV). A fertility plasmid, P, capable of mobilizing chromosomal genes has been identified and isolated in O1 V. cholerae (Bhaskaran, 1960; Datta et al., 1973) and used to construct a genetic map of the O1 serovar (Parker et al., 1979). A fertility factor, V, has been identified in a non-cholera vibrio strain (Bhaskaran & Sinha, 1971) and its behaviour has been studied in O1 strains. The V factor exhibits a lower rate of conjugative transfer than P and fails to mobilize chromosomal genes under the usual conditions of mating, although a very low level of gene transfer was demonstrated when V+ strains were incubated at 44.5 °C prior to mating (Bhaskaran & Sinha, 1971; Bhaskaran, 1975).

This paper presents evidence for the presence of factors similar to V and P in a non-O1 V. cholerae strain and an unidentified Vibrio strain of recent origin. Neither these factors nor the V factor could be isolated by standard plasmid isolation and detection procedures.

METHODS

Bacterial strains. Vibrio strains used are listed in Table I. Strains with the BV designation were obtained from W. M. Spira (Division of Geographic Medicine, School of Medicine, Johns Hopkins University, Baltimore, Md., USA) and are from a series of strains (BV2-BV93) isolated from aquatic sites in the environs of Dacca and Matlab, Bangladesh, in 1977 (McNicol et al., 1983). The BV strains used in this study, except BV13, were classified in an 83 unit character numerical taxonomy study (McNicol et al., 1983). BV93 was classified as V. mimicus by numerical taxonomy; however, BV93 DNA failed to hybridize with V. mimicus or V. cholerae DNA at the species level (H. Hada, personal communication). Strains were maintained at −70 °C in Difco nutrient broth containing 0.5%
<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Source, reference or derivation*</th>
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<tbody>
<tr>
<td>KB151</td>
<td><em>V. cholerae</em> O1, classical, P⁺, prototrophic</td>
<td>B. S. Srivastava</td>
</tr>
<tr>
<td>KB11</td>
<td><em>V. cholerae</em> O1, classical, P⁺, Ilv⁻ Arg⁻ His⁻ Str⁻</td>
<td>B. S. Srivastava</td>
</tr>
<tr>
<td>RJ3(pSJ5)</td>
<td><em>V. cholerae</em> O1, El Tor, arg-2 Str⁻, containing the P::Tnl hybrid Ap plasmid pSJ5</td>
<td>Johnson &amp; Romig (1979a)</td>
</tr>
<tr>
<td>NVC165</td>
<td>Non-cholera vibrio, V⁺, prototrophic</td>
<td>Bhaskaran &amp; Sinha (1971)</td>
</tr>
<tr>
<td>BV6, BV7, BV22, BV41</td>
<td>Non-O1 <em>V. cholerae</em>, prototrophic</td>
<td>McNicol et al. (1983)</td>
</tr>
<tr>
<td>BV11, BV12, BV93</td>
<td>Unidentified <em>Vibrio</em> sp., prototrophic</td>
<td>McNicol et al. (1983)</td>
</tr>
<tr>
<td>BV13</td>
<td><em>Vibrio</em>-like</td>
<td>W. M. Spira</td>
</tr>
<tr>
<td>VM5003</td>
<td>BV41, Cys⁻</td>
<td>Cys⁻ (ICR-191) mutant of BV41.</td>
</tr>
<tr>
<td>VM5048</td>
<td>BV22, His⁻ Str⁻</td>
<td>His⁻ (spontaneous) Str⁻ (spontaneous) mutant of BV22. This study.</td>
</tr>
<tr>
<td>VM5049</td>
<td>BV22, Phe⁻ Trp⁻ Str⁻</td>
<td>Phe⁻ Trp⁻ (ICR-191), Str⁻ (spontaneous) mutant of BV22. This study.</td>
</tr>
<tr>
<td>VM5050</td>
<td>BV22, Arg⁻†</td>
<td>Arginine sensitive (spontaneous) mutant of BV22. This study.</td>
</tr>
<tr>
<td>VM5051</td>
<td>BV12, Met⁻ Nal⁻</td>
<td>Met⁻ (spontaneous) Nal⁻ (spontaneous) mutant of BV12. This study.</td>
</tr>
<tr>
<td>VM5052</td>
<td>BV7, Arg⁻</td>
<td>Arg⁻ (spontaneous) mutant of BV7. This study.</td>
</tr>
</tbody>
</table>

* Addresses: B. S. Srivastava, Central Drug Research Institute, Lucknow, India; W. M. Spira, Johns Hopkins University, Baltimore, Md., USA. (ICR-191), obtained by ICR-191 mutagenesis; (spontaneous), obtained by spontaneous mutagenesis.
† Growth inhibited by arginine.

NaCl (NBS) and 12.5% (v/v) glycerol. All bacterial strains were routinely grown in NBS at 35 to 37 °C in a shaking water bath. Brain heart infusion broth (BHI; Difco) was used to obtain cultures of higher titres.

**Media.** Meat extract agar (MEA) and nutrient gelatin agar (NGA) were prepared as described by Parker & Romig (1972) except that 20 g Difco Bacto-agar l⁻¹ was used in MEA. The minimal medium used was that of Bhaskaran & Sinha (1971). Media was supplemented with antibiotics at the following concentrations (µg ml⁻¹): streptomycin, 50 to 100; ampicillin, 500; nalidixic acid, 20; kanamycin, 10. Amino acids were added at 20 µg ml⁻¹ and thiamin at 4 µg ml⁻¹.

**Mutagenesis.** Cells were treated with ICR-191 (Polyscience Inc.) as described by Roth (1971). Mutagenized cultures were enriched for auxotrophic mutants with nalidixic acid (Weiner et al., 1974).

**Quantitative lacunae assay.** This was done essentially as described by Parker & Romig (1972). Overnight cultures of test and indicator strains were diluted 10⁻² in BHI broth and incubated at 37 °C to a concentration of 10⁶ cells ml⁻¹. Test strains were incubated without aeration and indicator strains with shaking. About 10⁵ test strain cells and 10⁴ indicator strain cells were overlaid in NGA agar on MEA plates. Plates were held at ambient temperature for 15 min and incubated for 14 to 16 h at 37 °C. Lacunae appeared as circular areas of lesser turbidity against a background of confluent bacterial growth.

**Assay of lacunae and test strains for phage and bacteriocin.** Lacunae were stabbed with a sterile Pasteur pipette and adherent material was resuspended in 1 ml NBS. Suspensions were vortexed vigorously for 30 s, centrifuged at ambient temperature, and the clear portion of the supernate was recovered. Cultures of test strains were grown overnight at 37 °C and their supernates recovered as above. Lacunae and culture supernates were sterilized either by adding 0.05 ml chloroform or by centrifuging and recovering the supernate as described above two more times. Sterility was assayed by spreading undiluted samples on MEA plates. Samples (10 µl) of 10⁶, 10⁻¹, 10⁻² and 10⁻⁴ dilutions of the supernates (untreated and chloroform treated) were spotted directly on lawns of indicator cells. The plates were incubated for up to 48 h and periodically examined for lysis or inhibition of growth in the spotted areas. Lacunae and culture supernates were also assayed quantitatively for phage by substituting supernate for test cells in the standard lacunae assay. Lacunae and culture supernates of BV41 and BV93 were assayed on BV7, and those of NCV165 on BV22.

**Analysis of lacunae.** Lacunae were characterized as described by Parker & Romig (1972). Developed lacunae assay plates were inverted over a glass Petri dish lid containing chloroform and held for 2 to 3 h at 4 °C to kill the cells in the lawn, and then incubated with lids removed for 2 to 3 h to remove any traces of chloroform. Fresh NGA
Table 2. Quantitative lacunae assays

<table>
<thead>
<tr>
<th>Indicator strain</th>
<th>Percentage of cells producing lacunae</th>
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<tr>
<td></td>
<td>BV41</td>
</tr>
<tr>
<td>Non-O1 <em>V. cholerae</em> BV41</td>
<td>0</td>
</tr>
<tr>
<td>Non-O1 <em>V. cholerae</em> BV7</td>
<td>53</td>
</tr>
<tr>
<td>Unidentified <em>Vibrio</em> sp. BV11</td>
<td>49</td>
</tr>
<tr>
<td>Unidentified <em>Vibrio</em> sp. BV12</td>
<td>54</td>
</tr>
<tr>
<td><em>Vibrio</em>-like BV13</td>
<td>54</td>
</tr>
<tr>
<td>Non-O1 <em>V. cholerae</em> BV22</td>
<td>0</td>
</tr>
</tbody>
</table>

A agar inoculated with indicator cells was overlaid on the plates and the plates were incubated at 37°C overnight and scored for appearance of lacunae in the new lawn. Indicator strains were the same as those used for the assay of lacunae and test strains for phage and bacteriocin.

Serial fertility factor transfer. Cells within lacunae were streaked to single colonies on a rich medium. A master plate prepared from the single colonies was replicated to selective agar plates to distinguish between colonies of the indicator and test strain. Indicator colonies were assayed for lacunae production on a second indicator strain. Indicator colonies from these lacunae were selectively identified as above and tested for ability to produce lacunae on a third indicator strain.

DNA isolation. Extracts or lysates of chromosomal and plasmid DNAs were prepared by the method of Portnoy et al. (1981), Kado & Liu (1981), Newland et al. (1984) and Davis et al. (1980). Chromosomal and plasmid DNAs were isolated by ethidium bromide/caesium chloride density centrifugation of large scale plasmid extracts.

Restriction endonuclease digestions. These were done as recommended by the suppliers of the enzymes (BRL, Miles Laboratories and New England Biolabs).

Agarose gels. DNA was electrophoresed on horizontal 0.4% to 0.8% agarose (Sigma, Type I) gels, 5 mm thick, in Tris/acetate buffer, pH 8.0, at an approximate voltage of 2 V cm⁻¹.

Plasmid probe hybridization. Plasmid DNA was labelled by nick translation with [α-³²P]dATP (New England Nuclear) (Rigby et al., 1977; Maniatis et al., 1975). The probe was hybridized to plasmid and chromosomal DNAs by the method of Southern (Southern, 1975; Smith & Summers, 1980).

RESULTS

Assay for lacunae-producing ability

Initially, 24 strains identified as non-O1 *V. cholerae*, *V. mimicus* and *Vibrio* sp. isolated in the environs of Matlab, Bangladesh, in 1977 were assayed as indicator and/or test strains in a qualitative lacunae assay (Parker & Romig, 1972). Eight of the strains were tested for lacunae-producing ability and of these, BV41, a non-O1 *V. cholerae*, and BV93, an unclassified *Vibrio* strain, formed lacunae on some of the other strains. *V. cholerae* O1 strains KB11 and KB151, both containing the P plasmid, and the non-cholera vibrio strain NCV165, containing the V factor, also produced lacunae on some of the Bangladeshi strains. Results confirmed by quantitative assay are given in Table 2. KB11 and KB151 produced lacunae on non-O1 *V. cholerae* strains and on unidentified *Vibrio* strains. Approximately 50% of the test cells added to the assay plates gave rise to lacunae. Parker & Romig (1972) found that 50 to 100% of P⁺ *V. cholerae* cells produced lacunae on O1 *V. cholerae* indicators. BV41 and BV93 were similar to the P plasmid-containing strains in indicator hosts and percentage lacunae formation. NCV165 produced lacunae on the non-O1 *V. cholerae* strain BV22. The percentage of test cells producing lacunae was lower than that observed with the other test strains.

Nature of lacunae

The nature of the lacunae produced by NCV165, BV41, and BV93 was examined. To test for phage or bacteriocin mediation, sterile culture supernates of these strains were spotted on lawns of appropriate indicators. No inhibition of growth or lysis of the lawn cells was observed. Also, no lysis or plaques were observed upon quantitative plaque assay of the supernate solutions. Sterile supernates derived from lacunae produced by NCV165, BV41 and BV93 also failed to produce lysis of host cell lawns. Culture supernates and lacunae suspension supernates were
assayed with the same media and under the same conditions as the lacunae assay. Both chloroform-treated and untreated supernates were assayed to rule out the possibility that a phage or bacteriocin might be sensitive to chloroform.

Further evidence that a diffusible agent was not responsible for lacunae production was obtained by exposing plates containing lacunae to chloroform, to kill the cells in the lawn. Lacunae produced by NCV165 on indicator strain BV22 and by BV41 and BV93 on indicator strain BV7 were then analysed by overlaying the plates with a fresh indicator lawn. Following incubation, no lacunae were observed in the new lawn. Only when fresh test cells were added to the second overlay were lacunae observed, and these were at new sites in the overlay.

Serial transfer of lacunae-forming ability

Lacunae-forming ability of strains NCV165, BV41, and BV93 could be serially transferred to appropriate non-O1 V. cholerae and Vibrio sp. hosts. About 20% of host cells isolated from lacunae formed by NCV165 on VM5048 formed lacunae when used as test cells on VM5049. The same proportion of host cells isolated from the VM5049 indicator lacunae were then able to form lacunae on VM5050. In the same manner, lacunae-forming ability was transferred from a Cys− mutant of BV41 (BV5003) to VM5051 and thence to BV12, and from BV93 to VM5052 to BV7. In serial transfer of the factors in BV41 and BV93, about 12% of indicator cells isolated from lacunae were capable of forming lacunae on a new host.

Examination of strains for plasmid DNA

Strains BV41, BV93 and NCV165 were analysed for the presence of plasmid DNA. In these studies, single colony isolates of the strains verified as having lacunae-producing ability were used. Utilizing an 'in well lysis' technique for electrophoretic detection of plasmid DNA which can detect plasmids up to about 300 MDa (Newland et al., 1984), the 47 MDa P plasmid of strains KB11 and KB151 was detected, but no large plasmids were observed in strains BV41, BV93, or NCV165. Also, large plasmids could not be detected by electrophoretic analysis of plasmid extracts prepared by the methods of Kado & Liu (1981) and Portnoy et al. (1981). The conjugative R plasmid JR67 was transferred from an Escherichia coli host strain into strain NCV165 and a plasmid extract was prepared by the method of Kado & Liu (1981). The R plasmid was detected in the NCV165 host strain upon gel electrophoresis of the extract, indicating that failure to detect the V factor in strain NCV165 was not due to a general inability to detect plasmids in the host strain.

Large scale plasmid extracts, prepared by the method of Portnoy et al. (1981), and cleared Triton X lysates were subject to dye buoyant density centrifugation. Only a single DNA band was observed in gradients of BV41 and BV93, and the DNA migrated in gel electrophoresis at the position of chromosomal DNA. NCV165 gave two closely spaced DNA bands. These two bands were observed both with plasmid extract and cleared lysate preparations. The two bands, referred to as high density and low density bands, were collected, although due to their proximity they could not be cleanly separated. Both bands migrated on gels at the position of chromosomal DNA; however, upon digestion with PvuII restriction enzyme, several unique bands were observed with the high density DNA band fraction. Fig. 1 shows results of gel electrophoresis of the high and low density DNA bands along with plasmid and chromosomal DNA bands of strains KB11 and RJ3(pSJ5). The 25 MDa and 3 MDa cryptic plasmids reported in P+ classical genetic strains (Johnson & Romig, 1979b) were observed in plasmid DNA of KB11 but not in that of RJ3(pSJ5).

Hybridization with P plasmid DNA

Southern blots of the high and low density DNA bands of NCV165 and of the single chromosomal density gradient bands of BV41 and BV93 were probed with P plasmid DNA (Fig. 2). No hybridization of the probe DNA to NCV165, BV41 or BV93 DNA was observed. It therefore seems unlikely that plasmid DNA bearing homology to the P plasmid is banding in CsCl gradients with the chromosomal DNA of NCV165, BV41 or BV93.
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Fig. 1. Agarose gel electrophoresis of DNA isolated by ethidium bromide/CsCl density centrifugation of DNA extracts prepared by the method of Portnoy et al. (1981). Lane a, KB11 plasmid DNA containing the 47 MDa P plasmid and the 3 and 25 MDa cryptic plasmids. Lane b, KB11 chromosomal DNA. Lane c, RJ3(pSJ5) plasmid DNA. Lane d, RJ3(pSJ5) chromosomal DNA. Lane e, NCV165 low density DNA band. Lane f, NCV165 high density DNA band. Lane g, crude DNA extract of NCV165. The extra bands in lanes a and c represent the multimeric and linear forms of the plasmids.

Fig. 2. Southern blot of high and low density DNA bands of NCV165, and of chromosomal density gradient bands of BV41 and BV93. Lane a, λ digested with HindIII and ϕX174 digested with HaeIII. Lane b, BV41. Lane c, BV41 digested with XbaI. Lane d, BV93 digested with XbaI. Lane e, BV93. Lanes f and g, RJ3(pSJ5) plasmid DNA at two different concentrations. Lane h, KB11 plasmid DNA. Lane i, KB11 plasmid DNA digested with XbaI. Lane j, NCV165 high density DNA digested with XbaI. Lane k, NCV165 low density DNA digested with XbaI. Lane l, NCV165 high density DNA. Lane m, NCV165 low density DNA. The blot was overexposed to visualize any weakly hybridizing bands.
DISCUSSION

The lacunae assay, a phenotypic assay for the presence of P and V fertility factors (Bhaskaran, 1958; Bhaskaran & Sinha, 1971; Parker & Romig, 1972), was used to demonstrate the presence of similar factors, herein designated L factors, in a non-O1 V. cholerae strain, BV41, and in an unclassified Vibrio strain, BV93. The ability to produce lacunae was transferred to recipient (indicator) strains presumably via conjugation. Serial transfer of lacunae production indicated that lacunae-forming ability was not chromosomally encoded. Attempts to isolate an extracellular inhibitory substance such as a vibriocin or a phage from BV41, BV93 and the V factor-containing strain NCV165, or from lacunae produced by these strains, were unsuccessful. Parker & Romig (1972) studied lacunae produced by the P plasmid and concluded that they are most likely to be sites of active mating on the assay plates. Our results would support this interpretation for the lacunae produced by the V factor and the factors in BV41 and BV93.

The transfer properties of the V fertility factor have been studied previously (Bhaskaran & Sinha, 1971; Bhaskaran, 1975); however, there have been no reports of isolation of the V factor. We were unable to demonstrate the presence of plasmid DNA in the V factor-containing strain or in strains BV41 or BV93 by gel electrophoresis or dye buoyant centrifugation of plasmid extracts or lysates. Failure to detect plasmids in putative plasmid-containing strains has been reported and ascribed to several causes. The plasmid may exist largely integrated into the chromosome, as has been found for some conjugative R plasmids of Haemophilus influenzae (Stuy, 1980; Murphey-Corb et al., 1984). This is unlikely to be the case with the V and L factors since their transfer frequency is high. Hedges et al. (1975) were unable to isolate plasmid DNA from Pseudomonas strains harbouring plasmids of Inc group J by dye buoyant centrifugation. Since Rahal et al. (1978) found that only plasmids of Inc groups C and J are stably maintained in V. cholerae, the V and L factors may belong to Inc group J and, as found in Pseudomonas, be difficult to isolate.

Very large plasmids are difficult to isolate partly because of co-precipitation with chromosomal DNA in selective precipitation procedures (Hansen & Olsen, 1978). Such plasmids may also be sheared into linear fragments during extraction procedures and comigrate with chromosomal DNA in electrophoresis or dye buoyant centrifugation. V and P can stably coexist in the same cell line (Bhaskaran & Sinha, 1971), indicating that they belong to different incompatibility groups and hence should not display extensive DNA homology. However, it seemed reasonable to assume that V and P might have some gene region(s) in common since both factors are implicated in the production of lacunae, both promote low frequencies of chromosomal gene transfer and both inhibit production of cholera toxin in classical V. cholerae strains (Sinha & Srivastava, 1978a, b), and because the V factor inhibits the fertility of P (Bhaskaran & Sinha, 1971). Therefore, we probed the chromosomal fractions of plasmid extracts of NCV165, BV41 and BV93 with radiolabelled P DNA. Our failure to detect any hybridization indicates either that little or no linear plasmid DNA was present or that the P plasmid and the V factor share no homologous DNA sequences.

Our studies indicate that the V and L factors differ at the molecular level from the P plasmid, since, unlike P, these factors could not be detected or isolated by conventional plasmid techniques. In a survey of plasmid content of environmental and clinical strains of non-O1 V. cholerae, only small plasmids were found (Newland et al., 1984). Molecular definition of the V and L factors is needed to assess more fully the extrachromosomal elements in non-O1 V. cholerae and their role in the biology of these organisms.

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