Reversible expression of motility and flagella in *Clostridium chauvoei* and their relationship to virulence

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INTRODUCTION

Phase variation has been described for many prokaryotic systems (Silverman & Simon, 1980; Eisenstein, 1981; Meyers et al., 1982); perhaps the best characterized example is alternate expression of two distinct flagellar antigens in *Salmonella typhimurium* (Silverman & Simon, 1980). Phase variation of bacterial flagella has also been reported in other Gram-negative bacteria, such as *Pseudomonas aeruginosa* (Pitt, 1980), *Campylobacter jejuni* (Caldwell et al., 1985) and *Vibrio cholerae* (Mostow & Richardson, 1990). The phase change could be a defence mechanism to escape immune detection, as believed for *Salmonella* flagellar phase variation (Silverman & Simon, 1980), where the change in antigenic type helps the bacteria to evade the host immune system. Additionally, the ability to switch between flagellation and aflagellation may be an important virulence factor, since flagella appear either to protect *S. typhimurium* from the intracellular killing mechanisms of, or to enhance its ability to multiply within, murine macrophages (Weinstein et al., 1984, 1992; Tanaka et al., 1987; Kijima-Tanaka et al., 1994a, 1994b). However, there are still many points to be clarified in relation to determining whether the flagella of *C. chauvoei* are important as a virulence factor in the pathogenesis of infections caused by this organism.

While studying the role of flagella in pathogenesis, we observed that the normally motile *C. chauvoei* became spontaneously non-motile at a very high rate. Here we report our study on the reversible phase variation of motility and flagellation in *C. chauvoei* and its relationship to virulence.

METHODS

Bacterial strains. *C. chauvoei* strain Okinawa used in this study is widely used for vaccine production and as a standard challenge strain in potency assays in Japan (Azechi et al., 1962). Spores of strain Okinawa were produced according to the method of Bagadi (1977). The spore suspension in 10% (w/v) skimmed milk was lyophilized and stored at −20 °C until use. *C. chauvoei* ATCC 19399 was obtained from the American Type Culture Collection, Rockville, Md, USA.

Determination of transition rate. Both *Clostridium* strains were grown anaerobically on CLA agar (Azechi et al., 1962) supplemented with 5% (v/v) defibrinated sheep blood (CLA medium) at 37 °C until colonies were clearly visible, usually 18–24 h. Colonies were picked, grown in 1 ml CLA semi-solid medium (CLA medium containing 0-15% agar), and confirmed...
as motile by growth behaviour. Motile clones were detected by the fact that they grew into the medium from the line of inoculation whereas non-motile clones did not. Clones of a given phenotype harvested from CLA semi-solid medium were grown overnight on CLA medium at a density sufficient to give approximately 500 colonies per plate (usually 0·1 ml of a 10⁻² dilution per plate). The ratio of the number of variants to the total number of colonies gave the fraction of colonies which had undergone transition. This ratio was divided by the approximate number of generations the population had undergone to yield the transition rate per generation (Stocker, 1949). In calculating the number of generations, it was necessary to use cultures grown from a single cell to obtain a culture initially of a single phenotype. Presumptive single cell cultures were produced by inoculation with single colonies showing predominantly the required phenotype on CLA semi-solid agar. The number of generations the culture had gone through was calculated from the viable count at the time of testing.

**Antibodies.** For the plate agglutination test, anti-H serum and anti-O serum were prepared as described previously (Tamura et al., 1984). For Western blot analysis, polyclonal anti-serum to strain Okinawa and monoclonal antibody (mAb) Mo-114 were used. Mice received four intraperitoneal injections (0·25 ml), 2 d apart, of 0·4% formalin-treated whole cultures of strain Okinawa. Ten days after the final injection, mice were challenged intramuscularly with 2·3×10⁶ spores of strain Okinawa suspended in 0·25 ml 3% (w/v) calcium chloride solution. After 7 d, serum was collected and pooled (designated as anti-strain Okinawa serum). mAb Mo-114 against C. chauvoei flagella was prepared as described previously (Tanaka et al., 1987). This mAb recognizes a linear and internal epitope of the flagellar filament.

**Characterization of non-motile variants**

Each non-motile variant was examined for the following.

(i) **Biochemical properties.** The non-motile variants were characterized according to the description given by Cato et al. (1986). The production of hydrogen sulphide, indole, gelatinase, and acid from glucose, lactose, maltose, sucrose and salicin was tested in GAM (Gifu Anaerobic medium) semi-solid medium (Nissui). The production of lecithinase and lipase was tested in CW (Clostridium welchii) agar base (Nissui) supplemented with 4% (w/v) egg yolk and 8% skimmed milk. Antibiotic susceptibility was tested by an agar dilution method. Serial 10-fold dilutions of standard antibiotics supplied by the National Veterinary Assay Laboratory, Tokyo, Japan, were prepared so that concentrations of antibiotics ranged from 0·1 to 10 µg ml⁻¹. The antibiotics used were penicillin G, erythromycin, chloramphenicol and oxytetracycline.

(ii) **Serological properties.** Bacterial cells from an overnight culture on CLA medium were mixed with 20 µl suitably diluted anti-O serum and anti-H serum on a glass plate. The parental strain agglutinated fully within 1 min under these conditions.

**Electron microscopy.** Bacterial cells were cultured anaerobically on CLA medium at 37 °C for 24 h. The organisms were gently suspended in one drop 2% (w/v) ammonium acetate solution and placed on a collodion-carbon-coated grid. Cells were negatively stained for 1 min with 1% (w/v) phosphotungstic acid (TAAB Laboratories Equipment) and observed with a JEOL S-100 transmission electron microscope at 80 kV.

**Electrophoresis.** Purified flagella of strain Okinawa were prepared as described previously (Tamura et al., 1984) and suspended in 62·5 mM Tris/HCl buffer (pH 6·8) containing 2-mercaptoethanol (2%, v/v), urea (0·5 M), SDS (pH 6·8), glycerol (10%, v/v), and bromophenol blue (0·002%, w/v) at a concentration of 1 mg protein ml⁻¹. Suspensions of bacterial cells were prepared by inoculating 10 ml CLB medium with CLA semi-solid medium cultures of each bacterium. After incubation at 37 °C for 24 h, the broth cultures were centrifuged at 5000 g for 10 min. The cells were gently washed twice with Dulbecco’s phosphate buffered saline and suspended in the buffer described above. SDS-PAGE was performed with a Mini-Protean slab gel apparatus (Bio-Rad) with the buffer system of Laemmli (1970). Samples (10 µg) were stacked in 4% (w/v) polyacrylamide gels and separated in 12% polyacrylamide gels at 200 V for 45 min. Gels were stained with Coomassie brilliant blue R-250.

**Immunoblotting analysis.** Bacterial proteins were electrophoretically transferred to nitrocellulose membranes at 100 V for 1 h with a Trans-Blot apparatus (Bio-Rad) by the methanol-Tris/glycine system of Towbin et al. (1979). After being probed with mAb Mo-114 or anti-strain Okinawa mouse serum, the membranes were washed, incubated for 1 h with horseradish peroxidase-conjugated anti-mouse IgG goat serum, and washed again. They were then developed with horseradish peroxidase colour development reagent containing 4-chloro-1-naphthol (Bio-Rad).

**Virulence tests.** Six-week-old female outbred ddY mice (Nippon) were used. The virulence of C. chauvoei towards mice was determined as follows. After cultivation at 37 °C for 18 h on CLA semi-solid medium in the required phenotype, the test bacteria were subjected to serial 10-fold dilution to 10⁻²-fold with 3% calcium chloride solution. Calcium chloride was used to induce constant expression of the pathogenicity of C. chauvoei by stimulating germination of the spores and producing local tissue damage at the inoculation site. A portion (0·25 ml) of each 10-fold dilution of the bacterial suspension was injected intramuscularly into each of five mice. At the same time, 0·1 ml of each dilution was spread on CLA medium. Colonies were counted after anaerobic culture at 37 °C for 48 h. For determination of the 50% lethal dose (LD₅₀), mortality rates were recorded 7 d after injection and subjected to probit analysis. The determination of LD₅₀ values for each test bacterium was repeated twice.

**RESULTS**

**Detection of two colonial types**

Lyophilized spores of strain Okinawa were grown anaerobically on CLA medium at 37 °C for 24 h. By detailed observation, it was found that there were two types among the colonies (Fig. 1). One was large, low-

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**Fig. 1.** Two colonial types of C. chauvoei strain Okinawa. Large colonies (L) are formed by motile phenotypes, and small colonies (S) are formed by non-motile phenotypes.
convex, opaque, pale yellowish-grey; the other was small, low-convex, opaque, pale yellowish-grey. The large colonies were readily distinguished from the small colonies. The proportion of small colonies to large colonies was approximately 7:3. Since, in general, colony size is dependent on motility, the motility of large and small colonies was tested in CLA semi-solid medium. All large colonies tested had a motile phenotype as judged by growth behaviour, whereas all small colonies were non-motile. These colony and motility characteristics were maintained throughout subsequent subculture.

**Detection of spontaneous non-motile variants**

Since the lyophilized spores of strain Okinawa were prepared from pure cultures, it was possible that the above observations could be explained by variation from motile to non-motile phenotypes. Therefore, attempts were made to isolate the non-motile from the motile phenotype. Of 2984 colonies tested, 9 colonies had a non-motile phenotype at a rate of $2.16 \times 10^{-4}$. Strain ATCC 19399 was also screened and 3 of 1213 colonies tested had a non-motile phenotype at a rate of $1.79 \times 10^{-4}$. To determine the rate of reversion from non-motile to motile phenotype, the non-motile phenotype of strains Okinawa and ATCC 19399 were plated from a clonal population on CLA medium and after growth the colonies were examined for motility. Revertants to the motile phenotype were detected at a rate of $1.31 \times 10^{-4}$ and $2.39 \times 10^{-4}$, respectively.

**Characterization of non-motile variants**

Parent, non-motile variants and revertants of strain Okinawa were characterized (data not shown). The biochemical properties of every non-motile variant and revertant corresponded well with those of the parent. In serological tests with anti-O serum, all strains were agglutinated. With anti-H serum, parent and revertants were agglutinated, whereas three of nine non-motile variants were not. These results suggested that non-motile variants show both flagellate and aflagellate phenotypes.

**Electron microscopy**

To identify flagellar structures in non-motile variants, we examined negatively stained preparations of the cells by transmission electron microscopy. Three of the nine non-motile variants lacked a flagellar structure (Fig. 2b). In contrast, the six remaining non-motile variants did possess a flagellar structure (Fig. 2c) which was structurally indistinguishable from that of the parental strain (Fig. 2a).

**Electrophoretic analysis**

To determine whether there were other surface-related differences in addition to the presence or absence of flagella, the protein profiles of parent, and flagellate (designated M-F') and aflagellate (M-F-) non-motile variants and revertants were compared by SDS-PAGE (Fig. 3). The interpretation of a specific pattern was based on molecular masses rather than on the intensity of the bands. Protein patterns of all strains tested revealed the presence of approximately 35 bands, 12 of which were consistently prominent in Coomassie-brilliant-blue-stained gels. The major proteins had molecular masses of 115, 100, 93, 83, 74, 59, 52, 46, 40, 35, 17 and 12 kDa. No differences in the protein profile at a molecular mass of...
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Fig. 3. SDS-PAGE of *C. chauvoei* flagella and cells. Lanes: 1, purified flagella; 2, parental strain Okinawa; 3, non-motile aflagellate variant; 4, non-motile flagellate variant; 5, revertant. Gels were stained with Coomassie brilliant blue R-250.

Fig. 4. Immunoblotting of *C. chauvoei* flagella and cells with anti-strain Okinawa serum (a) and mAb Mo-114 (b). Lanes: 1, purified flagella; 2, parental strain Okinawa; 3, non-motile aflagellate variant; 4, non-motile flagellate variant; 5, revertant.

56 kDa, which corresponds to the flagellin monomer, were noted among the strains tested.

**Immunoblotting analysis**

Anti-strain Okinawa mouse serum and mAb Mo-114 directed to the flagella of *C. chauvoei* were used in immunoblots to detect structural proteins in cells of variants M⁺F⁻ and M⁺F⁺ (Fig. 4). Anti-strain Okinawa mouse serum reacted strongly with the protein bands of molecular mass 115, 93, 56 and 46 kDa from the preparations of all strains tested. In contrast, mAb Mo-114 reacted with the protein band of molecular mass 56 kDa, which corresponds to the flagellin monomer, from the preparations of the parent, M⁺F⁺ variants and revertants but not the M⁺F⁻ variants. The mAb Mo-114 also reacted with the 120 kDa band and two or more bands of 200 or more kDa, which are believed to be polymeric forms of the flagellin monomer, in purified flagella (Fig. 4b). These results confirmed that non-motile variants include both flagellate and aflagellate phenotypes. This distinction is not always easy to make by SDS-PAGE analysis.

**Determination of transition rate**

To demonstrate that M⁺F⁺ and M⁺F⁻ variants of strains Okinawa and ATCC 19399 were the result of spontaneous variation and to determine whether the phenomenon occurred in a strain other than Okinawa, we estimated the transition rate of both strains. The results are summarized in Table 1. These rates should be considered to be approximate because we observed some variability between experiments.

**Virulence studies**

To evaluate the role of motility and flagella in the virulence of *C. chauvoei*, mice were injected intramuscularly with clones of the two phenotypes. The results

### Table 1. Variation of motility and flagellation in *C. chauvoei*

<table>
<thead>
<tr>
<th>Strain</th>
<th>Variation</th>
<th>Total no. of colonies examined</th>
<th>No. of variants*</th>
<th>Transition rate per generation†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Okinawa</td>
<td>M⁺F⁻ → M⁺F⁺</td>
<td>2984</td>
<td>6</td>
<td>1·45 × 10⁻⁴</td>
</tr>
<tr>
<td></td>
<td>M⁺F⁻ → M⁺F⁻</td>
<td>2984</td>
<td>3</td>
<td>7·23 × 10⁻⁵</td>
</tr>
<tr>
<td></td>
<td>M⁺F⁺ → M⁺F⁺</td>
<td>839</td>
<td>13</td>
<td>0·96 × 10⁻³</td>
</tr>
<tr>
<td></td>
<td>M⁺F⁺ → M⁺F⁻</td>
<td>944</td>
<td>18</td>
<td>1·76 × 10⁻³</td>
</tr>
<tr>
<td>ATCC 19399</td>
<td>M⁺F⁺ → M⁺F⁻</td>
<td>1213</td>
<td>2</td>
<td>1·22 × 10⁻⁴</td>
</tr>
<tr>
<td></td>
<td>M⁺F⁻ → M⁺F⁻</td>
<td>1213</td>
<td>1</td>
<td>5·97 × 10⁻⁴</td>
</tr>
<tr>
<td></td>
<td>M⁺F⁻ → M⁺F⁺</td>
<td>2121</td>
<td>7</td>
<td>2·39 × 10⁻⁴</td>
</tr>
</tbody>
</table>

* Totals from several separate experiments.
† Ratio of the number of variants to the total number of colonies examined divided by the approximate number of generations the population had undergone (Stocker, 1949).
of these studies are summarized in Table 2. It was apparent from the investigations described above that the phenotype of the variants was essentially the same as that of the parents except for the loss of motility and in some cases, flagella. The flagellate phenotype was considerably more virulent than the aflagellate one, regardless of which clones were examined. There was no statistically significant difference in virulence between the M<sup>+</sup>F<sup>+</sup> variant and the parent. The similar LD<sub>50</sub> values of motile and non-motile phenotypes suggested that motility only very marginally affected virulence.

**DISCUSSION**

This study has shown that *C. chauvoei* strain Okinawa, which is used for the production of vaccine against blackleg, produced spontaneous non-motile variants. Three of nine non-motile variants were found to be flagellate, while the other six were aflagellate. These phenotypic variants occurred at a relative high frequency and showed biochemical and serological properties which were indistinguishable from those of the parental strains except for motility and flagellation. We also demonstrated reversible (in both directions) expression of motility and flagella in each variant. These data suggested that the ability to express both motility and flagella reversibly is inherent in the wild-type strain and not just a peculiarity of the original variants. Whether most strains of *C. chauvoei* are capable of this variation remains to be determined, but our preliminary observations indicated that motility and flagellar variation also occurs in *C. chauvoei* ATCC 19399. We concluded that transition and reversion of *C. chauvoei* flagella may be due to phase variation rather than mutation in view of its very high frequency, and also its oscillation between fixed alternatives which are characteristic of motility and flagellation. In general, phase variation of bacterial flagella has been found only in Gram-negative bacteria. It appears that the present work is the first report of the phenomenon in Gram-positive bacteria.

The flagella of *C. chauvoei* are involved in inducing immune resistance mechanisms, as demonstrated by the mouse protection test (Tamura *et al.*, 1984). Flagellar antibodies appear to exert this effect by opsonic activity such that opsonized *C. chauvoei* are eliminated by polymorphonuclear leucocytes (Tamura & Tanaka, 1984, 1987). Flagella have, therefore, been considered as one of the protective antigens contained in the vaccine against blackleg in domestic animals. Accordingly, this study provided additional information on the importance of seed control for the production of an effective vaccine against blackleg.

Flagella have also been implicated as virulence determinants in several enteric pathogens. A role for flagella in the adherence of *V. cholerae* to intestinal cells has been suggested (Attridge & Rowley, 1983; Guentzel & Berry, 1975). Flagella have been reported to enhance pathogenicity in *S. typhimurium* (Carsiotis *et al.*, 1984; Weinstein *et al.*, 1984), probably by increasing survival time within macrophages. Studies with colonization models have suggested that flagella play a role in the ability of *Campylobacter jejuni* to colonize the intestinal tract of animals (Newell *et al.*, 1985). Recently, it was reported that flagella are not involved in *C. jejuni* adherence to epithelial cells but that they do play a part in internalization (Grant *et al.*, 1993). Thus, the virulence of several bacterial pathogens in a variety of animal models and *in vitro* has been correlated with flagellation, chemotaxis and motility. In studies of bacterial virulence, a pair of isogenic strains would, by definition, differ only in the state of the single gene whose contribution to virulence is being assessed. With *C. chauvoei*, however, it has not been clear whether the flagella play an important role in infection.

To assess whether flagella and motility of *C. chauvoei* affected virulence in mice, we used phase variants believed to be isogenic with respect to loss of a single virulence factor in question. Parental strain Okinawa and flagellate variants were relatively virulent whereas aflagellate variants were nonvirulent. These results indicated that the flagella of *C. chauvoei* are associated with the expression of full virulence. Further studies on the role of flagella in infection of *C. chauvoei* are necessary in order to elucidate the pathogenicity of this species.

In the case of flagellar antigenic phase variation in *Salmonella* species, as with other examples of surface antigen phase variation, a programmed DNA rearrange-

### Table 2. Virulence of flagellate and aflagellate variants of *C. chauvoei* strain Okinawa in mice

<table>
<thead>
<tr>
<th>Clone</th>
<th>Phenotype</th>
<th>Motility</th>
<th>Flagellation</th>
<th>LD&lt;sub&gt;50&lt;/sub&gt;&lt;sup&gt;*&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Experiment 1</td>
<td>Experiment 2</td>
<td></td>
</tr>
<tr>
<td>6-1</td>
<td></td>
<td>&gt; 5.0</td>
<td>&gt; 5.4</td>
<td></td>
</tr>
<tr>
<td>5-12</td>
<td></td>
<td>&gt; 5.9</td>
<td>&gt; 5.3</td>
<td></td>
</tr>
<tr>
<td>5-11</td>
<td></td>
<td>0.2 (0.6 to -0.6)</td>
<td>1.0 (1.6 to -0.1)</td>
<td></td>
</tr>
<tr>
<td>5-14</td>
<td></td>
<td>1.0 (1.8 to -0.3)</td>
<td>0.8 (1.5 to -0.2)</td>
<td></td>
</tr>
<tr>
<td>Parent</td>
<td></td>
<td>0.2 (0.9 to -0.4)</td>
<td>0.6 (1.2 to -0.2)</td>
<td></td>
</tr>
</tbody>
</table>

* Expressed as the logarithm of the number of viable bacteria injected per mouse. Figures in parentheses indicate 95% confidence limit.
ment is involved (Eisenstein, 1981; Meier et al., 1985; Meyer et al., 1982). In J. typhimurium the rearrangement involves the reversible inversion of a 995 bp sequence 16 bp upstream from the gene which specifies H2 flagellin synthesis (Silverman & Simon, 1980). The molecular mechanism responsible for flagellar phase variation in C. chauvoei is unclear at this time, and will be the subject of future studies.

REFERENCES


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