Transposon-induced non-motile mutants of *Vibrio cholerae*

KATHLEEN RICHARDSON,^{1*} LINDA NIXON,¹ PETER MOSTOW,¹ JAMES B. KAPER²
and JANE MICHALSKI²

¹Department of Microbiology and Immunology, Oregon Health Sciences University, Portland, Oregon 97201, USA

²Center for Vaccine Development, University of Maryland School of Medicine, Baltimore, Maryland 21201, USA

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Non-motile mutants of *Vibrio cholerae* were isolated after transposon insertion mutagenesis with either Tn5 on a plasmid or Tn10ptac mini-kan in bacteriophage λ . The physical location and number of transposon insertions was determined. Eighteen Tn5 insertion mutants and 11 Tn10ptac mini-kan insertion mutants had single unique insertion sites. The 18 Tn5 insertions were contained within six different *Eco*RI fragments and the 11 Tn10ptac mini-kan insertions were contained within eight different fragments of *V. cholerae* chromosomal DNA. These data suggest that multiple genes are involved in motility. Immunoblot analysis of non-motile mutants with antibody to wild-type flagellar core protein indicated that two of the non-motile mutants made flagellar core protein. Three additional mutants reacted weakly with the antibodies. However, these mutants with immunopositive reactions did not produce any structures which resembled flagella by transmission electron microscopy. In addition, none of the other non-motile mutants produced wild-type flagella. However, five mutants which did not react in the immunoblot produced a structure which resembled a flagellar sheath without the internal flagellar core. In addition to having no filamentous core, the sheaths often extended from the sides of the bacteria, rather than from the poles where the flagellum is normally located. The data suggest that sheath formation is independent of flagellar filament formation, but that proper positioning of the sheath may require the flagellar filament.

Introduction

Motility of *Vibrio cholerae*, a Gram-negative rod-shaped bacterium, is due to a single, polar, sheathed flagellum. Although little is known about the function, composition, structure or genetics of this flagellum, the flagellar structure and composition appear to be more complex than those of *Escherichia coli* or *Salmonella typhimurium*. In addition to the four discs associated with all Gram-negative basal complexes, *V. cholerae* has a series of concentric rings which surround the flagellar axis and lie between the outer membrane and L ring and the peptidoglycan layer and P ring (Ferris *et al.*, 1984). Electron micrographs of *V. cholerae* flagella also show an electron-dense core surrounded by a membrane-like sheath which is contiguous with the bacterial outer membrane (Follet & Gordon, 1963). Several proteins (Hranitsky *et al.*, 1980) and lipopolysaccharide (Fuerst & Perry, 1988) are associated with the sheath. Purified

flagellar cores have been studied by polyacrylamide gel electrophoresis and immunoblotting with both polyclonal (Richardson & Parker, 1985) and monoclonal antibody (Harper *et al.*, 1985). Multiple proteins were observed, suggesting that the core is either composed of several distinct proteins or that *V. cholerae* flagellar cores can exhibit phase transition analogous to that seen in *S. typhimurium* (Macnab & Aizawa, 1984).

Previously, motility mutants of *V. cholerae* were obtained either spontaneously (Freter & O'Brien, 1981) or after chemical mutagenesis with agents such as *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) (Freter & O'Brien, 1981; Guentzel & Berry, 1975), a mutagen known to produce multiple mutations. Transposons have the advantage of producing defined mutations and have been used to obtain auxotrophic mutants of *V. cholerae* (Johnson & Romig, 1979; Newland *et al.*, 1984). Plasmid-borne transposons can be introduced into *V. cholerae* by conjugation. Additionally, since *V. cholerae* can be rendered susceptible to infection by bacteriophage λ (Harkki *et al.*, 1986), λ containing transposons (Way *et al.*, 1984) can also be used to mutagenize *V. cholerae*.

Abbreviations: NTG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; TB, tryptone broth; SDS-urea PAGE; sodium dodecyl sulphate-urea polyacrylamide gel electrophoresis; TEM, transmission electron microscopy.

Here, we report the isolation of non-motile *V. cholerae* mutants after transposon insertion mutagenesis. Our data suggest that multiple genes are involved in flagellar formation and that the sheath can assemble independently of the flagellar core.

Methods

Bacterial strains, plasmids, and bacteriophage. These are described in Table 1.

Transposon insertion mutagenesis with a F'_{is} plasmid vehicle. The donor strain, *E. coli* C600 containing the suicide vehicle $F'_{\text{is}}\text{lac}^+\text{Tn5Tn10}$ (Newland, 1984), was grown overnight at 30 °C in L-broth (Miller, 1972) containing 50 µg kanamycin ml⁻¹. The recipient strain, *V. cholerae* 395, was grown overnight in L-broth at 35 °C. A 0.1 ml volume of each overnight culture was mixed, spread on L-agar (Miller, 1972) and incubated at 30 °C for 3 h. Transconjugants were isolated by plating a portion of the mating mixture on L-agar containing polymyxin B (50 µg ml⁻¹) and kanamycin (50 µg ml⁻¹) and incubating at 30 °C. The presence of the plasmid in the transconjugants was confirmed by assaying for the Lac⁺ phenotype on MacConkey agar (Difco) containing lactose. *V. cholerae* containing the $F'_{\text{is}}\text{lac}^+\text{Tn5Tn10}$ plasmid could be maintained at 30 °C, stored frozen, and used repeatedly for mutagenesis. *V. cholerae* 395($F'_{\text{is}}\text{lac}^+\text{Tn5Tn10}$) was grown at 30 °C overnight in BHI broth (Difco), diluted 1:100 in 10 ml BHI and incubated at 40 °C for 6 to 8 h to obtain mutants with Tn5 insertions. The *E. coli* F plasmid is unstable in *V. cholerae* and the use of a vehicle which is temperature-sensitive for maintenance further promoted loss of $F'_{\text{is}}\text{lac}^+$ from *V. cholerae*. Furthermore, since *V. cholerae* are normally Lac⁻, the presence or absence of the plasmid was easily detected by growth on MacConkey agar.

Transposon mutagenesis with a λ vehicle. To make *V. cholerae* N16961 susceptible to infection by λ , plasmid pAMH62, containing *lamB* (*S. typhimurium* AS80), was mobilized into *V. cholerae* N16961 using the conjugative plasmid pRK2013 (in *E. coli* MM294). Each strain was grown overnight at 35 °C on L-agar containing the appropriate antibiotic (ampicillin for pAMH62 and kanamycin for pRK2013). Cells from each plate were mixed on a fresh L-agar plate and incubated overnight at 35 °C. A portion of this mating mixture was plated on MacConkey agar containing lactose, polymyxin B to counterselect the donors, and ampicillin to select for the *V. cholerae* transconjugants containing pAMH62. Lactose-negative, oxidase-positive colonies were then tested for motility in L-agar containing 0.6% agar (soft agar) and by phase-contrast light microscopy of an L-broth culture.

Cells were mutagenized using λ 1105 (Way *et al.*, 1984), containing Tn10 '*ptac*-transposase mini-kan', referred to as Tn10*ptac* mini-kan in this paper. *V. cholerae* N16961(pAMH62) was grown overnight with shaking at 30 °C in tryptone broth (TB) containing 10 mM-MgSO₄ and ampicillin (500 µg ml⁻¹). pAMH62, containing the *lamB* gene, is not stably maintained in *V. cholerae*. After overnight growth in L-broth initially containing 200 µg ampicillin ml⁻¹, only 23% of the *V. cholerae* contain pAMH62, as measured by expression of ampicillin resistance. To maintain a population of *V. cholerae* containing pAMH62 and thus susceptible to λ 1105 infection, we used high concentrations of ampicillin (500 µg ml⁻¹) during growth. The overnight culture was diluted 1:20 into 10 ml fresh TB containing MgSO₄ and ampicillin as above and incubated for 6 h at 35 °C with shaking. An additional 30 µg ampicillin was added to the culture to eliminate *V. cholerae* which did not contain pAMH62 and incubation continued for 30 min. The culture was then centrifuged and resuspended in 1 ml TB containing 10 mM-MgSO₄. λ 1105 at a multiplicity of infection of about 1 was added and the mixture incubated for 30 min at 25 °C. A 5 ml sample of L-broth,

pre-warmed to 35 °C, was added to the mutagenized cells and the mixture incubated for 90 min at 35 °C to allow for expression of kanamycin resistance. Curing the plasmid from mutagenized *V. cholerae* was easily accomplished by growing the mutants in the absence of ampicillin. In three experiments, we obtained an average frequency of 1×10^{-5} kanamycin-resistant *V. cholerae* per mutagenized *V. cholerae*. Of the kanamycin-resistant *V. cholerae* 0.1% were auxotrophic mutants.

Isolation of non-motile mutants. Mutagenized cells were plated in soft agar containing 50 µg kanamycin ml⁻¹. Non-motile cells gave rise to dense, pinpoint colonies, while motile *V. cholerae* formed haloed colonies. Pinpoint colonies were picked and streaked on L-agar containing kanamycin. Five to ten isolated colonies were then examined for motility by stabbing soft agar plates containing kanamycin and incubating at 35 °C. To distinguish non-motile mutants from chemotaxis mutants, presumptive non-motile mutants in L-broth were observed by phase-contrast light microscopy. Those mutants of *V. cholerae* strain 395 obtained from the $F'_{\text{is}}\text{lac}^+\text{Tn5Tn10}$ mutagenesis were also tested for loss of the plasmid by plating on MacConkey agar containing lactose. To test for insertion of only Tn5 and not Tn10, these mutants were plated on L-agar containing tetracycline (10 µg ml⁻¹).

Southern blot analysis. Chromosomal DNA from the non-motile mutants was isolated by the method of Silhavy *et al.* (1984). Chromosomal DNA (10 µg) was digested with *Cl*AI, *Eco*RI or *Sal*I (Bethesda Research Laboratories or Boehringer Mannheim Biochemicals) and electrophoresed on a 0.7% agarose gel. The DNA was blotted to nitrocellulose (Schleicher and Schuell) and reacted with probe DNA as described by Southern (1975). The probe DNA for the Tn5 insertion mutants was obtained from the plasmid pHU859 by restriction digestion with *Bgl*II, which cuts within Tn5 sequences to produce a specific probe, followed by agarose gel electrophoresis and recovery from DEAE-cellulose membrane (Schleicher and Schuell) using the manufacturer's protocol. The probe DNA for the Tn10 insertion mutants was isolated from the plasmid pNK862, which has the Tn10*ptac* mini-kan element inserted into a unique *Hind*III site. pNK862 was digested with *Hind*III and the Tn10 fragment purified in the same manner as for the Tn5 probe. The probe DNA was labelled with [α -³²P]dATP (New England Nuclear) by the random oligonucleotide primer protocol of Feinberg & Vogelstein (1983). Hexanucleotide triphosphates were obtained from Pharmacia. Unlabelled deoxy-nucleotide triphosphates were obtained from Boehringer Mannheim Biochemicals. Klenow PolI was obtained from Bethesda Research Laboratories. The labelled probes were recovered from the reaction mixture by precipitation with spermine (Sigma) according to the protocol of Hoopes & McClure (1981).

Immunoblot analysis. Wild-type *V. cholerae* and non-motile mutant strains were grown overnight in 1.5 ml L-broth at 35 °C. Cultures were centrifuged in microfuge tubes in an Eppendorf microfuge for 2 min, the supernatant removed and saved and the pellet resuspended in 100 µl L-broth. The cells were lysed by freeze-thawing 20 times, centrifuged for 2 min and the supernatant (cytosolic fraction) removed. The pellets of cells and envelopes were resuspended in 100 µl 1 × denaturing buffer (Richardson & Parker, 1985). The cytosol and culture supernatant fractions were each mixed with an equal volume of 2 × denaturing buffer. All samples were boiled for 5 min. Fractions were electrophoresed in sodium dodecyl sulphate-urea polyacrylamide gels (SDS-urea PAGE), electroblotted to nitrocellulose, and reacted with antiserum as described by Richardson & Parker (1985). The flagellar core proteins were purified as described by Richardson & Parker (1985), and antiserum was raised in a New Zealand White rabbit by repeated subscapular and intramuscular injection of 200 µg purified protein antigen in an equal volume of incomplete Freund's adjuvant. Pre-stained molecular mass standards (Bio-Rad) were run on

Table 1. *Strains, bacteriophage and plasmids*

Strain, phage or plasmid	Description or genotype	Source or reference*
<i>Vibrio cholerae</i>		
395	Classical, Ogawa; from patient in India	M. M. Levine
N16961	El Tor, Inaba; from patient in Bangladesh	M. M. Levine
KR1 to KR6 and KR8 to KR19	395 with <i>fla901::Tn5</i> to <i>fla906::Tn5</i> and <i>fla908::Tn5</i> to <i>fla919::Tn5</i>	This work
KR20 to KR30	N16961 with <i>fla920::Tn10-kanR</i> to <i>fla930::Tn10-kanR</i> †	This work
KR31	Non-motile, flagellate mutant of N16961; no transposon insertion	K. Richardson
KR1000	N16961 with <i>Tn10-kanR</i> insertion, auxotrophic	This work
<i>Escherichia coli</i>		
C600	<i>thr leuB6 thi supE44 lacY1 fhuA21</i>	
MM294	F ⁻ <i>endA1 thi-1 supE44 hsdR17</i>	
<i>Salmonella typhimurium</i>		
AS80	<i>his-6165 ilv-452 metA22 metE551 trpB2 galE496 xyl-404 rpsL120 flaA66 hsdL6 hsdSA29(malB)</i> from LT2	Harkki & Palva (1985)
Bacteriophage		
λ 1105	$\lambda::Tn10$ construct; 'ptac-transposase mini-kan' with <i>Tn10</i> insertion deleted for <i>tetR</i> , with <i>kanR</i> from <i>Tn903</i> inserted and transposase external to transposing element under control of <i>ptac</i>	Way <i>et al.</i> (1984)
Plasmids		
F _{ts} ⁺ <i>lac</i> ⁺ <i>Tn5Tn10</i>	<i>Tn5 kanR</i> , <i>Tn10 tetR</i> , <i>lac</i> ⁺ ; temperature sensitive for maintenance	Newland <i>et al.</i> (1984)
pAMH62	pBR322 derivative with <i>ompR'</i> <i>lamB</i> from <i>E. coli</i> inserted in <i>EcoRI</i> - <i>Bam</i> HI site, Ap ^r	Harkki & Palva (1985)
pNK862	pBR322 derivative with <i>Tn10</i> 'ptac-transposase mini-kan' construct in <i>EcoRI</i> site; Km ^r	Way <i>et al.</i> (1984)
pRK2013	Mobilizing plasmid; Km ^r	Figurski & Helinski (1979)
pHU859	pBR325::Tn5; Km ^r	R. Hull

* M. M. Levine, University of Maryland, Baltimore, Md, USA; R. Hull, Baylor, Houston, Texas, USA.

† *Tn10-kanR* corresponds to *Tn10ptac* mini-*kan*.

these gels. Pre-stained proteins exhibit altered migration due to covalent attachment of the dye. Pre-stained and un-stained standards were compared following SDS-urea PAGE, and apparent molecular masses calculated for each of the pre-stained standards.

Transmission electron microscopy (TEM). Wild-type *V. cholerae* and non-motile mutant strains were grown overnight in L-broth at 35 °C, diluted 1:100 in fresh L-broth, and incubated for 3 h at 35 °C. A drop of culture was placed on a 400-mesh parlodion-carbon coated grid (Structure Probe) for 1 min followed by a water wash. Cells were negatively stained for 1 min with 1% (w/v) uranyl acetate or 2% (w/v) phosphotungstic acid, pH 7.0. The grids were viewed in either a Philips 301 or a Siemens 1A transmission electron microscope at an accelerating voltage of 60 or 80 kV, respectively.

Reversion rate determination. Mutants were grown for 10 h at 35 °C in L-broth without kanamycin, diluted 1:100 in L-broth and incubated overnight at 35 °C with shaking. Cultures were centrifuged and resuspended in one-tenth the original volume of L-broth and 0.1 ml placed in a well cut in a soft agar plate without kanamycin. The plates were incubated at 35 °C overnight and observed for motile growth radiating out from the well.

Growth curves. Cultures grown for 16 to 18 h at 35 °C in L-broth were diluted 1:250 with 25 ml L-broth in a sidearm flask. The cultures were incubated at 35 °C with reciprocal shaking and the OD₅₅₀ determined at 30 min or 1 h intervals.

Oxidase reaction. To test for oxidase activity, cell paste from colonies on agar plates was smeared with a toothpick or platinum loop on a moistened oxidase strip prepared as follows: Whatman 3M paper was

saturated with a solution of 1% (w/v) *N,N,N',N'*-tetramethylparaphenylene diamine dihydrochloride (Sigma) and 0.2% (w/v) ascorbic acid in H₂O, dried and stored at 4 °C. If a dark purple coloration appeared within a few seconds of smearing the cell paste, the reaction was considered positive.

Results

Isolation and stability of non-motile mutants

The initial screening procedure in soft agar identified both presumptive non-chemotactic mutants and non-motile mutants. Thus, the mutants were confirmed as non-motile by light microscopy of wet mounts. For the *Tn10ptac* mini-*kan* insertion mutagenesis of strain N16961, mutation frequencies were 5×10^{-3} and 7×10^{-4} , respectively, for kanamycin-resistant, presumptive non-chemotactic mutants and non-motile mutants. The frequency of spontaneous non-motile mutants was less than 9.1×10^{-5} in strain N16961. All non-motile mutants in both strain 395 and strain N16961 were examined for reversion to the motile phenotype. Although the *Tn10ptac* mini-*kan* transposon insertion mutants should not revert since they lack the transposase gene (Way *et al.*, 1984), further transposition of *Tn5* in

Fig. 1

Fig. 2

Fig. 1. Analysis of Tn5 transposon insertion sites in non-motile mutants of *V. cholerae* 395. Isolated chromosomal DNA from each non-motile mutant was digested with *Eco*RI (panel I), which cleaves outside the Tn5 sequences, or *Sal*I (panel II), which cleaves once within Tn5. The fragments were separated by agarose gel electrophoresis, blotted and probed with Tn5. Lanes: A, KR4; B, KR16; C, KR5; D, KR15; E, KR17; M, pHU859 digested with *Sal*I. The molecular mass markers (M) 5.6 and 2.1 are *Sal*I fragments from pHU859 containing sequences of the Tn5 probe.

Fig. 2. Analysis of Tn10ptac mini-kan insertion sites in non-motile mutants of *V. cholerae* N16961. Chromosomal DNA from the non-motile mutants was digested with *Eco*RI (panel I), which cleaves outside the Tn10ptac mini-kan sequences, or *Cla*I (panel II), which cleaves once inside Tn10ptac mini-kan. The blot was probed with Tn10ptac mini-kan. Lanes: A, KR21; B, KR27; C, KR26; D, KR25; E, KR20.

strain 395 is possible. However, we did not detect any revertants of any transposon insertion mutant at our limits of detection (frequency about 1×10^{-11} per mutant).

Growth rate

The growth characteristics of the wild-type strains N16961 and 395, the Tn10ptac mini-kan control strain KR1000, and four non-motile mutants, KR10, KR12, KR26 and KR31, were examined to determine whether transposon insertion had any pleiotropic effects. The non-motile mutant KR31 was included as a control for the effect of motility on growth. This mutant does not contain a transposon insertion and was not obtained by

chemical mutagenesis. All the mutants grew with the same kinetics as their wild-type parental strains (data not shown). However, the two parental strains differed from each other in growth characteristics. The classical strain 395 and the mutant derivatives of 395 had a longer lag phase than did the wild-type and mutant derivatives of El Tor strain N16961. The growth rate in the exponential phase was the same for both biotypes.

Physical characterization of the transposon insertion mutants

Chromosomal DNA from each mutant was isolated, restricted, and analysed by Southern blotting to determine: (1) the number of transposon insertions in the

Fig. 3

Fig. 3. Transmission electron micrograph of the motile, flagellate, wild-type *V. cholerae* strains N16961 (a) and 395 (b) negatively stained with uranyl acetate. Original magnification of negative 10000 $\times$ for strain N16961 and 25000 $\times$ for strain 395. Bars, 500 nm.

Fig. 4

Fig. 4. Transmission electron micrograph of non-motile mutants of *V. cholerae*. (a) Aflagellate mutant KR3, negatively stained with uranyl acetate; original magnification of negative 19000 $\times$. (b) Non-motile mutant KR1 with a coreless sheath, negatively stained with phosphotungstic acid; original magnification of negative 14000 $\times$. Bars, 500 nm.

chromosome; (2) whether the insertions were in unique sites, and (3) the number of unique fragments that contained a transposon and therefore presumably gene(s) involved in motility. Chromosomal DNA was digested with either *EcoRI*, which does not cleave within either transposon sequence, or with a restriction enzyme that cleaves within the transposon, *SalI* for Tn5 and *ClaI* for Tn10ptac mini-kan. Figs 1 and 2 show the results of this analysis for several of the mutants. The Tn5 insertion into the *V. cholerae* strain 395 chromosome was random, based on Southern blot analysis of 26 non-motile mutants (Fig. 1) and 12 other uncharacterized Tn5 insertion mutants (data not shown). The Tn10ptac mini-kan also appeared to insert randomly into *V. cholerae* strain N16961, based on 14 non-motile mutants (Fig. 2) and 12 other uncharacterized Tn10ptac mini-kan insertion mutants (data not shown).

For the Tn5 insertion mutants, 25 of the original 26 non-motile mutants had single insertions; only single

insertion mutants were included in further studies. For the Tn10ptac mini-kan insertion mutants, all 14 non-motile mutants contained a single insertion of the transposon.

To identify unique insertion sites, we used two restriction enzymes, one which cuts within the transposon and one which cleaves outside the transposon, and did double digestions of the mutant chromosomal DNA. The migration of transposon-containing bands from non-motile mutants was compared (Figs 1 and 2, panels II; and data not shown). Of the 25 non-motile Tn5 insertion mutants, 18 had distinct banding patterns, indicating different Tn5 insertion sites. Eleven of the 14 Tn10ptac mini-kan insertion mutants had distinctly unique insertions. Based on the Southern analysis, we identified six unique *EcoRI* fragments containing Tn5 ranging in size from about 8 to 36 kb, and eight unique *EcoRI* fragments containing Tn10ptac mini-kan, ranging in size from about 3 to 36 kb. Thus, at least eight and

Table 2. Characteristics of motility mutants

Mutant designation	Morphology*		Immunoblot reactivity (45–50 kDa)†
	Fla	Sheath	
Classical			
395 (wild-type)	+	+	+
KR1	–	+	–
KR2	–	+	–
KR4	–	+	–
KR15	–	+	–
KR16	–	+	–
KR3, KR5, KR6; KR8–KR14; KR17–KR19	–	–	–
El Tor			
N16961 (wild-type)	+	+	+
KR20	–	–	+
KR22	–	–	+/-
KR23	–	–	+/-
KR24	–	–	+/-
KR26	–	–	+
KR21, KR25; KR27–KR30	–	–	–

* The presence of the flagellar structure (Fla) or sheath was determined by TEM of negatively stained bacterial preparations.

† Three preparations, cytosol, membrane and supernatant fractions, from each mutant and parental strain were separated by SDS-urea PAGE and immunoblotted with antiserum to the flagellar core proteins. Three classes of bands were observed: an 83 kDa band, four bands migrating with apparent molecular masses of 45 to 50 kDa (flagellin), and a 26 kDa band. +/- indicates that very faint bands were observed.

possibly 14 *Eco*RI fragments of chromosomal DNA contain genes involved in motility.

Electron microscopy of the non-motile mutants

We examined negatively-stained preparations of all non-motile mutants with single transposon insertions by TEM (Table 2). The wild-type flagellum for strains N16961 and 395 is shown in Fig. 3. None of the non-motile mutants had the typical wild-type flagellum, and most were aflagellate (Fig. 4a). However, five non-motile mutants of strain 395 had a structure that appeared to be a flagellar sheath without the flagellar core (Fig. 4b). No coreless sheath mutants of strain N16961 were observed. In these coreless sheath mutants, the sheath-like structure was present on 50% of the cells. In contrast to the wild-type flagellum, which always extends from the pole of the bacterium, the sheaths of these mutants generally extended from the side and only rarely (less than 1% of coreless sheaths observed) from the pole.

Immunoblot analysis for flagellar core proteins

Membranes, cell cytosol, and cell supernatants from all 29 mutants with single transposon insertions were separated by SDS-urea PAGE and probed with antiserum to *V. cholerae* flagellar core proteins (Table 2). In the motile control strain, KR1000, the serum reacted with four bands (apparent molecular masses 45 to 50 kDa) from the cytosol and membrane fractions, but not from the supernatant fraction (Fig. 5, sample 1). Two of the bands reacted weakly. Results from representative mutants are shown in Fig. 5, samples 2–8. Normal flagellar core proteins were observed in the membrane and cytosol fractions from two non-motile, aflagellate mutants, KR20 (Fig. 5, sample 2) and KR26 (sample 5). Three non-motile, aflagellate mutants (KR22, KR23, and KR24) reacted weakly with the serum (data not shown). No flagellar core proteins were detected in any of the 18 mutants of strain 395 or the other six mutants of strain N16961 (Fig. 5, samples 3, 4, 6, 7 and 8, and data not shown), including the five mutants with coreless sheaths (Fig. 5, sample 7). A high molecular mass band (apparent molecular mass 83 kDa) was seen in the membrane fraction from 14 of the mutants of strain 395, including all of the coreless sheath producing mutants; in 10 of the mutants of strain N16961; and in flagellate strains (just visible in samples 1, 2 and 7). This band is probably a cross-reactive antigen or, less likely, a flagellar core protein precursor. For six mutants of strain 395 and four mutants of strain N16961 a low molecular mass band was seen (apparent molecular mass 26 kDa; Fig. 5, samples 3, 4 and 5, and data not shown). This band was never seen with preparations from strains which made an intact flagellum.

Discussion

We used two different vehicles, Tn5 on a plasmid and Tn10*ptac* mini-kan in a bacteriophage, to obtain non-motile mutants of classical (strain 395) and El Tor (strain N16961) *V. cholerae*, respectively. By TEM, none of the mutants produced typical flagella. Based on analysis of electron micrographs and production of flagellar core proteins as determined by immunoblots, the 29 mutants could be divided into three phenotypes: five mutants had at least some flagellar core protein in their membranes; four mutants produced sheath-like appendages without any core protein; and the remaining mutants produced neither the sheath-like appendages nor any core protein (Table 2).

We do not know whether the reactivity to bands with apparent molecular masses of 83 kDa and 26 kDa represents reactivity to flagellin precursors and breakdown products or cross-reactivity with heterologous

Fig. 5. SDS-urea PAGE and immunoblot analysis of *V. cholerae* motility mutants probed with antiserum to *V. cholerae* flagellar core protein. For each strain, cell fractions of supernatant (S), cytosol (C), and membrane (M) were examined; all three lanes are labelled only for sample 1. Sample 1, KR1000 (flagellate control); samples 2-8, non-motile mutants - 2, KR20; 3, KR11; 4, KR25; 5, KR26; 6, KR3; 7, KR16 (coreless-sheath mutant); 8, KR21. Values on the right indicate the migration distances of pre-stained protein standards: 97.4, phosphorylase *b*; 68, bovine serum albumin; 43, ovalbumin; 25.7, α -chymotrypsin; 18.4, β -galactosidase.

proteins. Isolated flagellar cores when examined by SDS-urea PAGE exhibit only the four bands with apparent molecular masses of 45 to 50 kDa (Harper *et al.*, 1985; K. Richardson, unpublished). The absence of the 26 kDa band from *V. cholerae* producing normal flagella suggests that this band may represent degraded flagellin protein. Flagellin made by aflagellate mutants may be degraded in the cytoplasm. It is curious that only a single band is observed when four proteins are associated with the intact flagellar core.

We isolated five mutants that do not make intact flagella, by TEM analysis, but do produce flagellar core proteins, based on the immunoblot analysis. The transposon insertions which produce this phenotype occur in three different *EcoRI* fragments. The genes present on these fragments may be regulatory genes or encode products necessary for transport or assembly of flagellar core protein.

We also isolated four mutants with aberrant flagella (Fig. 4*b*). Published electron micrographs of normally sheathed flagella (Follett & Gordon, 1963; Sjobald *et al.*, 1983) suggest that these aberrant flagella are membranous sheaths without an internal filamentous core. The overall structure of these coreless sheaths is elongated and extends from the outer membrane as does a normal sheath. However, the diameter of these sheaths is irregular and rarely extends from the pole of the bacterium. Thus, it appears that sheath assembly is independent of core formation but that the core structure may be essential for normal sheath morphology.

It is interesting that the coreless sheath mutants were only isolated from the classical strain of *V. cholerae* and that production of flagellin protein in aflagellate mutants only occurred in derivatives of the El Tor strain. These results could reflect unique attributes of the two strains, N16961 and 395, chosen for these studies or be due to the use of two different transposons to mutagenize each strain. The two biotypes of *V. cholerae* differ from each other in many ways. The different mutant phenotypes we observed for each biotype may reflect further differences that distinguish these two biotypes.

Analysis of the physical location of the transposon insertion in the mutants allowed us to ascertain that each mutant contained a single unique insertion. In addition, the Southern analysis indicated there are at least eight *EcoRI* fragments of chromosomal DNA which contain flagellar genes. The location and arrangement of *V. cholerae* flagellar genes on the chromosome is only known for one mutant locus, *mot-1* (Parker *et al.*, 1979); *E. coli* and *S. typhimurium* flagellar and motility genes are arranged in several operons clustered into three regions on the chromosome (Macnab & Aizawa, 1984). *Pseudomonas aeruginosa* flagellar mutations appear to be clustered in two regions (Tsuda & Iino, 1983). However for *Caulobacter crescentus*, while most of the flagellar and motility genes map in three clusters at widely separated regions of the chromosome, several other genes are scattered around the chromosome (Ely *et al.*, 1984). We observed that insertions in a single *EcoRI* fragment gave mutants with at least two different phenotypes, i.e.

aflagellate and flagella consisting of a coreless sheath, suggesting that some of the *V. cholerae* flagellar genes are clustered. However, whether the *V. cholerae* flagellar genes are arranged on the chromosome like those of *E. coli* or *C. crescentus* cannot be inferred from these results.

The non-motile mutants isolated in this study were all without typical flagella. This phenotype could result from polar effects of transposon insertion on expression of either genes within the same operon or genes in separate operons. Expression of flagellin genes in *E. coli* is tightly regulated. Mutations in any one of the approximately 30 flagellar genes arranged in six separate operons results in a non-motile, aflagellate phenotype (Macnab & Aizawa, 1984). We have not yet determined whether the lack of flagellum formation observed in the non-motile *V. cholerae* mutants described here is due to polar effects of the transposon insertion in a system similar to that of *E. coli*. Further studies of these mutants will clarify the organization and expression of *V. cholerae* flagellar genes.

Many molecular vectors and techniques used with *E. coli* can be readily adapted for *V. cholerae*. Both the Tn5 and Tn10ptac mini-kan insertions in the chromosome occurred predominantly as single events and appeared to be random. Although we did not observe any revertants of the Tn5 insertion mutants, an additional advantage of using Tn10ptac mini-kan for mutagenesis is that this transposon cannot undergo any further transposition since the transposase gene is left behind during transposition from λ to the chromosome. However, to mutagenize *V. cholerae* with λ 1105 as the vehicle a λ receptor must be expressed from a cloned gene and form a functional receptor on the bacterial surface. The use of $F'_{ts}lac^+$ as the vehicle for mutagenesis does not require additional factors, since *V. cholerae* readily transfers plasmids by conjugation. Finally, *V. cholerae* containing the $F'_{ts}lac$ plasmid vehicle can be stored frozen or lyophilized, grown at 30 °C, and when further mutants are desired the culture is simply grown at the non-permissive temperature. A distinct advantage of using λ 1105 for mutagenesis is that the bacteriophage vehicle for Tn10ptac mini-kan cannot replicate in *V. cholerae*, hence virtually all kanamycin-resistant *V. cholerae* have the transposon in the chromosome. In contrast, the $F'_{ts}lac^+$ plasmid vehicle segregates out as a result of growth at the non-permissive temperature. Antibiotic-resistant isolates obtained following growth at the non-permissive temperature must be examined for persistence of the plasmid vehicle. Additionally, the period of incubation following mutagenesis with λ 1105 is shorter than that used with $F'_{ts}lac^+$, so the potential for isolating mutants that are siblings of each other is reduced.

Although we successfully used bacteriophage λ as a vehicle for introducing DNA into *V. cholerae* and

obtained mutants, we note that mutagenesis with Tn10ptac mini-kan via the λ 1105 vehicle has worked erratically. Despite examination of the various components of the system, none appears to be clearly responsible. Variable mutation frequencies were obtained using preparations of λ 1105 produced at different times and in separate laboratories. Using a preparation that had mutagenized *V. cholerae* very well, several λ 1105 preparations were obtained from single plaques. While these single-plaque preparations mutagenized *E. coli* at high frequency (1×10^{-4} per *E. coli* mutagenized), they mutated *V. cholerae* very poorly. By analysis of restriction enzyme sites, pAMH62 purified from *V. cholerae* was identical to that from the original *S. typhimurium* strain. We also have demonstrated by immunoblot analysis that *lamB* is expressed and the gene product localized to the outer membrane of *V. cholerae*. However, in *V. cholerae* the receptor does not appear to be associated with the peptidoglycan in the same manner as in *E. coli* (unpublished observations), since no high molecular mass species are detected from *V. cholerae*.

We have isolated and characterized 25 non-motile mutants in a classical strain of *V. cholerae* and 11 non-motile mutants in an El Tor strain of *V. cholerae* produced by transposon insertion mutagenesis. Further genetic studies using these mutants should elucidate the arrangement of *V. cholerae* flagellar genes on the chromosome, their expression, and their regulation. These non-motile mutants will also be useful in examining the role of motility and flagellar structures in pathogenesis of cholera and the survival of *V. cholerae* in the estuarine environment.

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