Colonial opacity variations among the cholera vibrios

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Cultures of *Vibrio cholerae* O1, biotype El Tor, from the current epidemic of cholera in the Western Hemisphere, and of the new *V. cholerae* serogroup O139, from the current outbreak in India and Bangladesh, revealed marked colonial heterogeneity when received by the authors. By comparison with reference colony types, using a stereoscope and transmitted oblique illumination, colonies of approximately 10 different degrees of opacity could be distinguished. In contrast, strains freshly isolated from patients and rapidly and carefully preserved were more homogeneous although still differentiable by this technique. These (and older) observations prompted the questions: (1) why is a *V. cholerae* colony opaque or translucent? and (2) what benefit is it to the vibrios to vary their colonial appearance? The observed changes in colonial opacity, which are reversible, are sometimes (rarely) accompanied by changes in virulence for infant rabbits and, more frequently, by other phenotypic variations including the ability to produce poly- β -hydroxybutyrate inclusion bodies on glycerol-containing medium, the degree of encapsulation in O139, changes in outer-membrane proteins, alteration in lipopolysaccharide structure, changes in expression of glycolytic pathways, and differences in ability to survive under adverse conditions. Colonial variations in cholera vibrios are phenotypically multifactorial. The genetic mechanisms(s) underlying the observed phenotypic changes remain to be defined.

Keywords: *Vibrio cholerae*, colonial variation, opacity, poly- β -hydroxybutyrate (PHB), capsules

INTRODUCTION

Cholera vibrios have long been known to be mercurial in their ability to exhibit variations in colonial morphology. Robert Koch was the first to observe that the colonial appearance of cholera vibrios was distinct from that of other bacterial species (Pollitzer, 1959, p. 125). Soon thereafter, Kolle and Gotschlich (Pollitzer, 1959, pp. 452–456), and subsequently others, noted that vibrio subcultures contained opaque (O) and translucent (T) colonial variants. Lankford (1960) observed that nearly all colonies of classical biotype *Vibrio cholerae* on primary culture from patients in Calcutta, India, in 1953, and in Bangkok, Thailand, in 1958, were of a single characteristic 'typically opaque and striated' colony type, whereas most stock cultures of *V. cholerae*

were mixtures of colony types or pure types differing from each other. Colonies which deviated from the 'typical' colony type usually had reduced virulence for 13-d-old chicken embryos (Lankford, 1960; Gardner *et al.*, 1964). The apparent homogeneity of fresh isolates has also been the author's (R.A.F.'s) experience during cholera epidemics of El Tor and classical biotypes in the Philippines, India, Vietnam and Thailand during the period from 1961 to 1967. In 1966, it was noted (Finkelstein, 1966) that stock cultures of El Tor biotype *V. cholerae* sent from Tehran, Iran, yielded O and T colonies, of which the T types were haemolytic and the Os were not. This was at a time when the El Tor vibrios, the cause of the present seventh great cholera pandemic, were losing their haemolytic ability which was, originally, the *sine qua non* for their differentiation from the classical biotype. Subsequently, it was observed (Holmes *et al.*, 1975) that the virulent hypertoxinogenic T colony type of El Tor biotype Ogawa serotype strain 3083

Abbreviations: LOS, lipooligosaccharide; MR, methyl red; OU, opacity unit; PHB, poly- β -hydroxybutyrate; VP, Voges-Proskauer.

spontaneously and reversibly produced O colonial variants which were Tox⁻ and avirulent. These observations have been repeated with that strain in our laboratory many times subsequently (M. Boesman-Finkelstein, unpublished observations), although it was noted recently (Finkelstein *et al.*, 1992) that other opacity/translucency changes were not accompanied by changes in virulence and toxinogenicity either in strain 3083 or in strains recently received from Peru. Cultures of the new *V. cholerae* serogroup O139 (syn. Bengal) from the current outbreak in India and Bangladesh (Albert, 1994), recently received and examined in our laboratory, were found to be markedly heterogeneous. We therefore decided to address the questions of the mechanisms and reasons for colonial variation among the choleraenic vibrios.

In other micro-organisms (Anderson & Dawes, 1990; Johnson *et al.*, 1992; Mills *et al.*, 1992; Page & Knosp, 1989; Poolman *et al.*, 1985; Swanson *et al.*, 1992; Weiser, 1993; Weiser *et al.*, 1994; Wise *et al.*, 1992; Yoshida *et al.*, 1985), colonial variations such as those noted above have been associated with a variety of properties including changes in content of poly- β -hydroxybutyrate (PHB), changes in outer-membrane proteins, lipoproteins, lipopolysaccharides (LPS) or lipooligosaccharides, capsulation or piliation, which may or may not be associated with changes in virulence in pathogenic species. In a recent study of *Haemophilus influenzae* by Weiser *et al.* (1995), in addition to differences in LPS structure and encapsulation observed previously between opaque and less opaque colonial phenotypes, a novel 47 kDa opacity-associated protein, OapA, was identified which was associated with the translucent phenotype and ability to colonize the nasopharynx (of mice) but not with virulence by intra-peritoneal challenge.

Although relative little is known about these phenomena in *V. cholerae*, Lankford (1960) had alluded to a correlation between opacity and the presence of sudanophilic lipid globules within the cells and, more recently, strains of *V. cholerae* O139 were reported (Johnson *et al.*, 1994; Weintraub *et al.*, 1994) to have a polysaccharide capsule which correlated with opacity.

Portions of the work described here have been presented at the General Meeting of the American Society for Microbiology, 23–27 May 1994 (Las Vegas, NV), and at the US–Japan Cooperative Medical Science Program, 31st Joint Conference on Cholera and Related Diarrheal Diseases, Kiawah Island (Charleston), South Carolina, 1–3 December 1995.

METHODS

Vibrio strains, media, and procedures. *V. cholerae* O1 strains of El Tor and classical biotypes and Inaba and Ogawa serotypes were primarily from our own culture collection maintained by lyophilization since shortly after isolation or receipt and, as working stocks, by storage at -70°C in tryptic soy broth with 20% (v/v) glycerol. Serogroup O139 cultures from the current outbreak in India and Bangladesh were

provided by G. Balakrish Nair and M. John Albert, respectively (Shimada *et al.*, 1993). M. John Albert also provided strains, isolated in 1994 and 1995, which were lyophilized from the progeny of isolated single colonies from the primary culture of the patients' specimens (stool or rectal swab) on TCBS agar. Y. Takeda provided strain O139 M045 and unabsorbed rabbit antisera to it (Shimada *et al.*, 1993).

Routinely, Petri dishes, 85 mm diameter, containing 16 ml meat extract agar (MEA; 0.3% Bacto beef extract, 1.0% Bacto-peptone, 0.5% NaCl and 2% Difco agar) or modified '2-1 agar' (Henry, 1933; Braun, 1953) – the medium used previously by Lankford (1960) – a formulation similar to MEA but containing 0.5% Bacto beef extract and 0.5% glycerol, were streaked for semi-confluent growth and incubated at 37°C for 16 h, harvested with 10 ml sterile 0.1% peptone saline and decimally serially diluted. Ten microlitre droplets of a 1:2 dilution of the 10^{-6} dilution were applied to previously dried duplicate Petri dishes and allowed to dry in so that no visible surface moisture remained before incubation. This procedure may be expected to yield approximately 25 isolated colonies in a circumscribed area about 1 cm in diameter. Standard reference strains arbitrarily defined from previous experience as representing 2, 4, 6 and 8 opacity units (OU) were deposited in a square pattern in the centre of the Petri dish and unknown cultures, treated similarly, were dropped around the periphery (see Fig. 1). Observations were usually made after 16–18 h incubation at 37°C using a stereoscope and transmitted oblique illumination according to a modification (Finkelstein & Punyashthiti, 1967) of the technique of Henry (Braun, 1953). For evaluation of variability, multiple observers were given brief instructions and asked to grade the opacity of the unknowns by comparison with the reference strains. Means and standard errors were then calculated. One O139 strain, MDO-12 from Madurai, India, which, when received by us, yielded three colony types of increasing opacity, MDO-12-A, MDO-12-B, and MDO-12-C, was selected for more extensive study. Strain MDO-12-C forms the most opaque *V. cholerae* colonies we have seen (OU = 10).

Image analysis and processing. A stereoscope equipped with a black-and-white CCD (charge-coupled device) camera was used to capture digital images of individual colonies growing on agar plates. Along with each colony image, an image of an adjacent region of agar without a bacterial colony was acquired to serve as a measure of background density. The Image1 AT (Universal Imaging) image analysis system was used to subtract the background (agar only) density from the colony's image and quantify the area brightness (average pixel brightness values) of the colonies. A CCD equipped with a macroscopic lens was used to capture the images of whole plates used as illustrations in this report. To accentuate the differences in grey-scale opacity, software (Adobe Photoshop) was used to create an arbitrary colour look-up table to pseudo-colour the digitized images.

Transmission electron microscopy. Bacteria growing as isolated colonies were overlaid with molten 2% agar in PBS and immediately chilled on a bed of ice. The plate was then covered with 10 ml paraformaldehyde/glutaraldehyde fixative (PFG; 2% freshly depolymerized paraformaldehyde, 2.5% glutaraldehyde, 70 mM NaCl, 30 mM HEPES, 2 mM CaCl_2 , pH 7.4) for 3 h at 4°C . A razor blade was then used to cut out thin ($1 \times 1 \times 2$ mm) rectangles of bacterial colonies sandwiched between the two agar layers. Following overnight fixation in fresh PFG, the samples were rinsed eight times in HEPES washing buffer (HWB; 70 mM NaCl, 30 mM HEPES,

pH 7.4) over the course of 2 h. The samples were post-fixed in 1% osmium tetroxide in HWB for 4 h at room temperature, rinsed six times with deionized water, and *en bloc* stained in 0.5% uranyl acetate for 1 h. Following extensive rinsing, the samples were dehydrated with a series of ethanol and propylene oxide and embedded in EmBed812 epoxy resin. Thin sections were stained with uranyl acetate for 10 min and lead citrate for 3 min before examination in a transmission electron microscope.

PHB staining. In some experiments, 0.5% malachite green hydrochloride was added to the PFG fixative to selectively stain the PHB granules (Kushnaryov *et al.*, 1980; Sun *et al.*, 1994). All other steps followed our routine fixation and embedding procedure.

PHB analysis. PHB content of the bacterial cells was analysed by HPLC after acid hydrolysis to crotonic acid as described by Karr *et al.* (1983). Wet cells, ranging from 100 to 200 mg, harvested from semi-confluently streaked Petri dishes, were digested in 1 ml concentrated sulfuric acid at 90 °C for 30 min and then cooled on ice. (Lyophilized or oven-dried, 80 °C overnight, cells were used for dry weight determinations.) Before analysis by HPLC, digested samples were appropriately diluted with 7 mM H₂SO₄ and filtered through a 0.45 µm cellulose acetate filter (Costar Europe) to remove particulate material. Samples (50 µl) were then injected into a fast acid analysis column (100 × 7.8 mm; Bio-Rad) and eluted with 7 mM H₂SO₄ at a flow rate of 0.5 ml min⁻¹. Absorbance of crotonic acid was then measured at 214 nm (Millipore Waters Lambda-Max model 481, LC spectrophotometer). The amount of crotonic acid produced was determined from the area of the peak of a known crotonic acid standard. Finally, on the basis of stoichiometry of the chemical reaction, the amount of crotonic acid produced was expressed in terms of PHB. It should be noted that PHB values may fluctuate depending on the age of the culture, confluency of the bacterial growth, and variations in processing.

PHB and longer-chain β-hydroxyalkanoates, especially hydroxyvalerate, were determined using washed and then dried (lyophilized) bacteria by the method of Page *et al.* (1992), except that the polymer was not purified before methanolysis, but instead, was extracted from the cells with chloroform. Parallel studies with *Azotobacter vinelandii* cells indicated that this method extracted approximately 50% of the polymer.

Capsule staining. Capsules were demonstrated by staining with polycationic ferritin (Johnson *et al.*, 1992). Agar-grown bacteria were flooded with 5% glutaraldehyde in HWB, pH 7.0, for 90 min at room temperature. The bacteria were then resuspended in the same fixative using gentle trituration with a Pasteur pipette and placed in a 15 ml centrifuge tube for an additional 30 min of fixation. After gentle centrifugation and resuspension in HWB three times to remove the fixative, the bacteria were exposed to polycationic ferritin (1 mg ml⁻¹; Sigma) in HWB for 2 h at room temperature and then washed in the same buffer three times. The final pellet was resuspended in 0.3 ml ice-cold buffer and mixed with 0.5 ml 2% agar in HWB at 42 °C in a 15 mm wide well of a 24-cell tissue culture tray sitting on ice. The fixed agar disk was removed, diced into 1 × 2 × 2 mm rectangles and fixed in 1% osmium tetroxide in HEPES buffer overnight. Following washing, the agar pieces were dehydrated and embedded in epoxy resin as described above. Thin sections were stained with uranyl acetate for 10 min and lead citrate for 3 min before examination in a transmission electron microscope.

Outer membrane preparation. Outer membranes were prepared from different *V. cholerae* strains by treatment with sarcosine as described by Filip *et al.* (1973) with minor modifications. Bacteria were first deflagellated by homogenization and then disrupted by an ultrasonic disintegrator at 4 °C. Unlysed cells were then removed by low-speed centrifugation (8000 g, 10 min) and the crude envelope fraction was collected from the supernatant by ultracentrifugation (100000 g, 1 h). The pellet containing the crude envelope fraction was treated with 0.5% (w/v) Sarkosyl (Sigma) solution to selectively solubilize the inner membrane part. The insoluble outer membrane fraction was finally recovered as pellet by ultracentrifugation (100000 g, 1 h) at 4 °C. The pellet was washed once with Sarkosyl (0.5%) solution and once with distilled water and finally stored at -20 °C until used. Protein content of outer-membrane preparations was determined by the method of Markwell *et al.* (1978) with bovine serum albumin as a standard.

LPS preparation. LPS was prepared from whole-cell *V. cholerae* lysates by the proteinase K digestion method as described by Kimura & Hansen (1986). Bacteria were first lysed in 0.5 vol. concentrated digestion buffer [0.1875 M Tris/HCl, pH 6.8; 6% (w/v) SDS; 30% glycerol] at 100 °C for 5 min. The bacterial lysates were then diluted fourfold with LPS digestion buffer (0.0625 M Tris/HCl, pH 6.8; 0.1% SDS; 10% glycerol and 0.005% bromophenol blue) and deproteinized by proteinase K (Sigma).

SDS-PAGE. Samples were analysed on SDS-polyacrylamide gels [12.5% (w/v) for protein and 15% (w/v) for LPS] using 4.5% (w/v) stacking gels on top (Laemmli, 1970). Electrophoresis was carried out at room temperature at constant voltage (120 V) until the tracking dye reached a point 1 cm above the bottom of the separating gel. After electrophoresis, proteins and LPS were visualized by the silver staining methods of Oakley *et al.* (1980) and Tsai & Frasch (1982), respectively.

Frequency of colonial variation. The frequency of colonial variation was determined for exponential-phase cultures of single-colony isolates of strains MDO-12-A, MDO-12-C, and 3083-O in tryptic soy broth using the formula: frequency = [(no. of variants)/(total viable cell count)]/no. of generations.

Survival under adverse conditions. A preliminary effort was made to evaluate the relationship, if any, of colonial opacity to survival under adverse conditions. Previous experience (Finkelstein, 1962) had demonstrated that cholera vibrios tend to die off relatively rapidly when suspended in normal saline but were protected by a concentration of peptone, 0.1%, which was not sufficient to support multiplication. Accordingly, suspensions of approximately 10⁶ viable cells ml⁻¹ were incubated in normal saline for 24 h at 4, 25 and 37 °C, and dilution-plated to determine viability.

RESULTS

Stereoscopic observations with transmitted oblique illumination

To begin the present study, we examined a variety of isolated colonies of laboratory strains of *V. cholerae* O1 and O139 grown on meat extract agar (MEA) using a stereoscope and transmitted oblique illumination as described previously (Finkelstein & Punyashthiti, 1967). We could easily classify unknown cultures into approximately 10 different degrees of opacity by comparison

Fig. 1. Visual images of *V. cholerae* O139 colonies growing on nutrient agar supplemented with either 0% (a) or 0.5% glycerol (b). The centre colonies, clockwise from top left, are four reference strains: 1852 O (OU = 8), 4450 T (OU = 6), 1855 T (OU = 2) and 1838 T (OU = 4). The peripheral colonies are, clockwise from the top: MDO-12-B (OU = 4), MDO-12-A (OU = 2), MO 45 (ranked at OU = 7 with a few more translucent colonies) and MDO-12-C (OU = 10).

with reference cultures (Fig. 1a). Individuals with no previous experience could quantify the opacity of cultures with a high degree of reproducibility. However, whereas we have previously (Finkelstein & Gomez, 1963; Finkelstein & Punyashthiti, 1967) regarded freshly isolated wild-type *V. cholerae* as being extremely translucent when compared with colonies of other intestinal microflora on MEA, Lankford (1960) regarded the *V. cholerae* colonies he isolated in Calcutta and in Bangkok as 'typical opaque striated' and noted that a small proportion of less opaque or even translucent colonies sometimes appeared transiently in the primary cultures. In static or serially transferred cultures (Lankford, 1960), the population became progressively less opaque and more variable. One significant difference between Lankford's and our work was that Lankford used '2-1 agar', a nutrient agar medium of similar composition to MEA but which had a higher concentration of beef extract, 0.5%, and also contained 2% glycerol and 1% glucose (Henry, 1933; Braun, 1953). Using the same reference cultures, a series of experiments (results not shown) revealed that it was the glycerol in Lankford's medium that affected the opacity of the colonies. Although the relative order of opacity of the reference colonies was maintained, the differences between the more translucent and the more opaque colonies were enhanced on glycerol-containing medium (compare Fig. 1a and Fig. 1b). Furthermore, 0.5% glycerol (Fig. 1b) was found to be optimal in this regard and therefore this formulation was used in our subsequent studies.

The visual observations were highly reproducible to different individuals. Average transmitted light intensities, determined with the Image-1 analysis system, were found to be proportional to visual observations, but not always in perfect agreement, suggesting that other optical qualities (e.g. tint and opalescence) that could be discriminated by the human eye but not by image analysis were involved.

PHB

Because sudanophilic lipid had earlier been implicated in opacity of *V. cholerae* (Lankford, 1960) and PHB had been observed in *V. cholerae* and other vibrios (Hood *et*

al., 1986; Sun *et al.*, 1994; Tamura *et al.*, 1968) and associated with opacity in other bacteria (Anderson & Dawes, 1990; Page & Knosp, 1989), we undertook to examine the PHB content of various strains and colonial variants of *V. cholerae* using electron microscopy and biochemical analysis.

Electron microscopy. When MDO-12-C was grown on 0.5% glycerol-containing medium for 12 h, cross-sectional profiles of bacteria were frequently observed to contain one to four electron-dense membrane-bound granules which were stained by the PHB-specific dye malachite green. Opaque bacteria also contained 10–20 electron-translucent inclusion bodies (Fig. 2a).

Both the malachite-green-stainable granules and electron-translucent granules were still present in individual colonies of MDO-12-C grown for 24 h (Fig. 2b) although confluent lawns of MDO-12-C were found to contain neither type of inclusion body after 24 h (not shown). At 36 h, individual colonies of MDO-12-C had reduced content of both types of inclusion body (Fig. 2c). The granules were apparently consumed under crowded conditions and with ageing of the culture. When MDO-12-C was grown without glycerol for 12 h, only occasional examples of bacterial cross-sections contained a malachite-green-stained granule; these granules were smaller and more electron dense than ones observed in colonies grown on 0.5% glycerol (compare Fig. 2d with Fig. 2a). In colonies of the translucent colony type MDO-12-A grown on 0.5% glycerol for 12 h, there was no evidence of either the malachite-green-stained granules or the electron-translucent granules (Fig. 2e). Bacteria in individual colonies of the intermediate opacity strain MDO-12-B occasionally contained a small densely stained malachite-green-stained granule but few, if any, of the electron-translucent granules (Fig. 2f). Interestingly, in MDO-12-B, the malachite-green-stained granules were frequently located along the periphery of the cell as opposed to the more centrally located granules of MDO-12-C. Occasional examples of extracellular malachite-green-stained granules were found in the MDO-12-B colonies but not in MDO-12-A or MDO-12-C colonies.

Biochemical analysis. As shown in Table 1, the PHB

Fig. 2. Malachite-green-stained transmission electron micrographs of: (a) MDO-12-C, 12 h + 0.5% glycerol; (b) MDO-12-C, 24 h + 0.5% glycerol; (c) MDO-12-C, 36 h + 0.5% glycerol; (d) MDO-12-C, 12 h + 0% glycerol; (e) MDO-12-A, 12 h + 0.5% glycerol; (f) MDO-12-B, 12 h + 0.5% glycerol. Large arrows, malachite-green-stained granules; small arrows, electron-translucent inclusions. Bar, 1 μ m.

contents of translucent variants (MDO-12-A and MDO-12-B) of strain MDO-12 were low, even in the presence of glycerol, whereas the PHB content of opaque variant MDO-12-C peaked sharply at concentrations of 0.5–1.0% glycerol to levels more than 10 times those of the translucent variants on 0.5% glycerol. Analyses of a large number of fresh isolates, carefully preserved, of both O1 and O139 serogroups on glycerol medium revealed (Table 2, Fig. 3) a strong and significant correlation between opacity and the ability to produce PHB.

It is interesting to note that the freshly isolated O139 strains fell into three opacity groups, with OUs ranging from 8.1 to 8.4, 7.4 to 7.6, and 6.4 to 6.9 (Table 2a), which would not have been recognized as different if they had been observed on different plates and not compared with reference colonies. Strain AJ 27-439-T was from a translucent sector found in the growth of its parent strain AJ 27-439, which had 2.6-fold higher PHB content. Most of the O1 El Tor Ogawa strains included in Fig. 3 were less opaque and produced less PHB than the O139 strains and they also seemed to fall into three

Table 1. PHB content of variants of *V. cholerae* O139 (syn. Bengal) strain MDO-12 grown for 12 h on nutrient agar with varying glycerol concentrations

Variant	Glycerol (%)	PHB*
MDO-12-A	0.5	2.5†
MDO-12-B	0.5	4.0†
MDO-12-C	0.0	3.6‡
	0.25	5.4‡
	0.5	26.4§
	1.0	26.9‡
	2.0	18.3‡

* Determined as crotonic acid (Karr *et al.*, 1983); mg (g dry wt)⁻¹.

† Result of two identical independent determinations.

‡ Result of a single determination.

§ Mean of three independent determinations (range 20.0–32.5).

groups, OU 6.1–6.7, 5.2–5.6 and 4.3–4.7 (Table 2b). When similar studies were performed with a random collection of 34 older stock cultures and colonial variants (Table 3), the correlation, although significant, was not as striking ($r^2 = 0.41$; data not shown). Although the sample size is small, the older classical biotype O1 isolates seem to have higher PHB levels. As a general rule, strains with OU ≤ 5 had significantly lower PHB content than strains with OU > 5 on glycerol-containing medium (Table 4).

Four strains, CA-411, CA-172, CA-174 and CA-401, from Lankford's original collection of classical biotype *V. cholerae* isolated in Calcutta in 1953 (Lankford, 1960) which had been regarded by him as 'typical opaque', and preserved by lyophilization since that time, were scored between 5.2 and 5.6 OUs and had PHB contents ranging from 31.2 to 47.2 mg g⁻¹ (Table 3). Strain 3083-T, a variant selected from an El Tor biotype culture isolated (by R.A.F.) in Vietnam in 1963 and preserved carefully since then, formed the most translucent colonies we have seen (OU = 1) and was virulent and toxinogenic whereas a more opaque (OU = 5.3) colony type isolated from the same strain was avirulent (Holmes *et al.*, 1975). On glycerol medium, these two had PHB contents of 9.3 and 17.6 mg g⁻¹, respectively. It is interesting that these two can be differentiated by the naked eye and, before the advent of the O139 isolates and our use of glycerol medium, we had regarded 3083-O as unusually opaque. It appears that the ability to produce PHB, while related to opacity in the absence of glycerol and contributory to it in its presence, is not directly or totally responsible for opacity – especially when one considers the MDO-12 series, cited above, which were originally graded by their opacity on glycerol-free medium in which their PHB contents were low. However, the addition of glycerol markedly expands the range of opacity which is observed.

Polyhydroxyalkanoate analyses by the method of Page

Table 2. Opacity and PHB content of 'single-passage' *V. cholerae* O139 (a) and O1 strains (b) from Bangladesh

Strain	Opacity (OU)*	PHB†
(a) O139 strains		
AJ 27-513	8.4 (0.24)	37.1
-495	8.4 (0.24)	33.2
-493	8.2 (0.20)	29.5
-448	8.4 (0.24)	31.2
-426	8.2 (0.20)	29.5
-425	8.4 (0.24)	21.7
150649‡	8.1 (0.20)	34.0
AJ 27-514	7.6 (0.24)	26.8
-439	7.4 (0.24)	22.7
1433569‡	7.6 (0.16)	25.9
AJ 27-404	6.5 (0.32)	18.1
-370	6.4 (0.24)	17.1
150648‡	6.7 (0.17)	19.2
1653‡	6.8 (0.17)	19.1
1433574‡	6.9 (0.21)	19.4
AJ 27-439-T§	5.0 (0.0)	8.8
(b) O1 strains		
143390‡	6.7 (0.25)	12.2
150640‡	6.6 (0.20)	10.9
150678‡	6.4 (0.23)	10.3
1718‡	6.1 (0.17)	12.9
1434625‡	5.6 (0.20)	9.8
150659‡	5.2 (0.12)	8.3
1434450‡	4.7 (0.25)	2.3
150668‡	4.4 (0.33)	2.3
1433875‡	4.3 (0.33)	2.3
20492‡	4.3 (0.31)	1.9

* Mean ($\pm$ S.E.), as determined by five observers, of colonies on glycerol (0.5%) medium in comparison with reference opacity standard strains.

† Determined as crotonic acid (Karr *et al.*, 1983); mg (g dry weight)⁻¹. Mean of duplicate assays of 18 h grown bacteria.

‡ Isolates from August 1995. Other strains were from October 1994.

§ Colonial (T) variant of AJ 27-439.

et al. (1992) confirmed that PHB was consumed in older cultures of MDO-12-C. The results also indicated that *V. cholerae* forms a small amount of hydroxyvalerate (HV), most likely in the form of the common copolymer P (HB-co-HV) in which the HV content is quite low, i.e. approximately 4–5 mol %. MDO-12-A, the most translucent variant, formed neither PHB nor PHV detectable in this assay.

Capsulation

Reports of capsulation of *Vibrio vulnificus* and of non-O1 *V. cholerae* (including O139 strains) and its association with opacity prompted us to examine the

Fig. 3. PHB content of freshly isolated and carefully preserved strains of (○) *V. cholerae* O139 and (●) O1 (El Tor Biotype) from Bangladesh.

presence of capsules in the colonial variants of strain MDO-12. The most opaque colony type, MDO-12-C, was heavily encapsulated as revealed by electron microscopy with polycationic ferritin (Fig. 4a): it was surrounded by a thick deposit of cationized ferritin presumably defining the termini of long-chain capsular polysaccharides. Its capsules were much more massive than those reported by others (Johnson *et al.*, 1994; Weintraub *et al.*, 1994). The more translucent strain MDO-12-B (Fig. 4b) had a smaller coat of cationized ferritin closer to the outer membrane which was similar to previous reports. The most translucent strain, MDO-12-A, was coated with a thin layer of cationized ferritin lying close to the outer membrane (Fig. 4c). Such a staining pattern is consistent with binding to the non-capsular LPS and the absence of long-chain capsular polysaccharide.

Virulence

Unlike strains 3083-T and 3083-O described previously (Holmes *et al.*, 1975; M. Boesman-Finkelstein, unpublished results), MDO-12-A and C were both found to be toxinogenic and equally virulent for infant rabbits. Choleraenic scores (Finkelstein *et al.*, 1992), at 24 h, for three infant rabbits per group inoculated intraintestinally with 10^7 live vibrios of MDO-12-A and MDO-12-C, were 7.0 and 6.7, respectively.

Outer-membrane proteins

Colonial variants of both O139 and O1 serogroups also exhibit changes in outer-membrane proteins (OMPs) (Fig. 5a). Translucent strain 3083-T has a 49 kDa OMP (Fig. 5a; compare lanes 1 and 2) which is absent in the more opaque 3083-O. Translucent variants MDO-12-A and B express a 27 kDa OMP which is not present in opaque MDO-12-C (Fig. 5a; compare lanes 4 and 5 with lane 3). We have demonstrated that on switching to

and fro between translucent and opaque forms these proteins are lost and regained (unpublished data).

Lipopolysaccharide/lipooligosaccharide

Changes in LPS structure (Fig. 5b) [or, as the LPS does not exhibit the ladder structure typical of highly polymerized simple sugars, the term lipooligosaccharide (LOS) may be preferable] were also observed in colonial variants of MDO-12. MDO-12-C (Fig. 5b, lane 3) produces a slower-migrating LOS than MDO-12-A and B (Fig. 5b, lanes 1 and 2), resembling that of El Tor biotype strain 3083 (Fig. 5b, lanes 4 and 5). These structures are similar to the multiple electrophoretic forms of the O139 antigen described earlier (Waldor *et al.*, 1994).

Methyl red/Voges-Proskauer

Because of the extremely wide range in opacity observed in colonies from cultures which were sent to us from other areas, the identity of unusually opaque or translucent colonies was confirmed by slide agglutination tests with specific antisera and by biochemical tests used taxonomically. All were confirmed to be as labelled – even the extreme variations noted in culture MDO-12. Interestingly, however, whereas – like most El Tor biotype strains – MDO-12-C was Voges-Proskauer (VP) positive and methyl red (MR) negative (i.e. it produced acetyl methyl carbinol and did not utilize the mixed acid fermentation pathway), MDO-12-A and B gave the reverse pattern. That these variants arose from a single strain, i.e. that they are reversible, was demonstrated by testing variants from cultures established from serial passage of single colonies (unpublished observations).

Frequency of colonial variation

The frequencies of colonial variation determined from exponential-phase cultures of single-colony isolates of strains MDO-12-A, MDO-12-B and 3083-O were determined to be 9×10^{-4} , 4×10^{-4} and 1×10^{-3} per bacterium per generation, respectively. In broth, variation of cultures grown to stationary phase was more frequent in static than in serially passed cultures, suggesting that accumulation of metabolites or deprivation of nutrients may facilitate enrichment of aberrant colony types. It should be mentioned that the MR⁺ translucent strains produced very tiny colonies on the original 2-1 agar formulation, which contained 1% glucose; perhaps, because of their mixed acid fermentation pathway, they were inhibited by acid production in the presence of glucose.

Survival

Finally, to approach the question of the potential advantage of colonial heterogeneity to *V. cholerae*, representative colony types, harvested at 18 h from MEA and 0.5% glycerol agar, were diluted to approximately 10^6 viable cells ml⁻¹ in normal saline incubated

Table 3. Opacity and PHB content of *V. cholerae* strains and colony types from various sources

Serovar	Source (year)	Strain	Opacity (OU)	PHB§
O139	Bangladesh ('93)	1837-T	7.0	25.0
		1838-T†	4.0	2.3
		-O	5.0	2.1
		1841	3.0	2.5
		1852-O†	8.0	22.3
		1855-T†	2.0	2.1
		-O	5.0	2.3
		4450-T†	6.0	38.5
		4460-T	6.0	25.0
		-O	9.0	23.4
	India ('93)	MDO-12-A	2.0	1.8
		-B	4.0	2.6
		-C	10.0	37.5
		PO2	5.0	2.1
		MO-45-T	5.0	1.8
		AP1-3	9.0	30.9
		AP2-2	7.0	19.4
O1 (ET-O)	Philippines ('61)	26-3‡	6.4	23.7
	('65)	65-7-5-T	2.4	3.9
		-O	8.0	26.8
	Vietnam ('63)	3064-1‡	5.8	18.8
		3083-T‡	1.0	9.3
		-O‡	5.3	17.6
		3148-11‡	5.8	23.9
O1 (ET-I)	Korat, Thailand ('66)	HP-30‡	5.3	23.0
		HP-71‡	5.2	23.1
	Port Lavaca, TX ('73)	3242-73	5.5	14.8
		2469-T ('86)	4.0	1.9
O1 (C-O)	India ('41)	NIH-41	6.8	16.7
		CA-411‡	5.6	31.2
		CA-172‡	5.5	47.2
		CA-174‡	5.5	30.4
O1 (C-I)	('53)	NIH-35-A3	6.4	30.9
		CA-401‡	5.2	32.3

* ET-O, El Tor biotype, Ogawa serotype; ET-I, El Tor biotype, Inaba serotype; C-O, classical biotype, Ogawa serotype; C-I, classical biotype, Inaba serotype.

† Arbitrarily selected to be opacity reference standards for 2, 4, 6, and 8 OUs.

‡ Lyophilized (by R.A.F.) shortly after isolation from patient. Other strains were maintained in other laboratories and one to three colonies were selected by us when they were streaked in our laboratory. The designations, T and O, were in reference to each other at that time. OUs were determined subsequently by comparison with reference standard strains.

§ Determined as crotonic acid (mg per g dry weight of bacteria grown for 15–16 h on medium containing 0.5% glycerol).

for 24 h at various temperatures (Table 5). In practically every instance, survival for 24 h was higher with the more opaque variants – in some instances the differences in survival were 10-fold or more. The opaque encapsulated MDO-12-C had the highest survivability but the differences between 3083-O and 3083-T were noteworthy. When grown in the absence of glycerol,

cells of the MDO-12 series survived less well in normal saline than when they were grown in glycerol, but the protective effect of the capsule now became evident. One may conclude that both PHB levels and amount of capsule can contribute to survival under adverse conditions. In contrast to the recent observations of Ravel *et al.* (1995) in 'artificial seawater', no reversion to viability

Fig. 4. Polycationic-ferritin-stained transmission electron microscopy of: (a) MDO-12-C; (b) MDO-12-B; and (c) MDO-12-A. Bar, 0.5 μ m.

Table 4. PHB content of *V. cholerae* O1 and O139 strains of varying opacity (data from Table 3)

Opacity		PHB†	
Range*	Mean $\pm$ SE	Range	Mean $\pm$ SE
1–5 (12)	3.5 $\pm$ 0.4	1.8–9.3	2.9 $\pm$ 0.6
> 5–10 (22)	6.6 $\pm$ 0.3	14.8–47.2	26.4 $\pm$ 1.7

* Number of strains examined shown in parentheses.

was observed when the temperature was shifted up from 4 °C to 30 °C.

DISCUSSION

Faced by many previous observations of colonial heterogeneity which, in some instances, were associated with such important phenotypic properties as virulence for chicken embryos (Lankford, 1960; Gardner *et al.*, 1964), haemolysis in the O1 El Tor biotype (Finkelstein, 1966), toxigenicity and virulence (Holmes *et al.*, 1975), and, most recently, capsulation in the new O139 serovar (Johnson *et al.*, 1994; Weintraub *et al.*, 1994), we decided to attempt to define the reason(s) for opacity and translucency and the potential value of such variations to the vibrios. The present findings suggest that colony opacity variations among the cholerae vibrios are multifactorial. It should be noted that freshly isolated and carefully preserved strains are considerably more homogeneous than stock cultures; i.e. we may conclude that a great deal of the heterogeneity we have observed occurs subsequent to primary isolation.

Our first step was to develop a reproducible method of quantifying opacity. This was accomplished by defining arbitrarily a set of reference opacity standard strains with which to compare unknown cultures on the same Petri dish using transmitted oblique illumination and a stereoscopic microscope. With this technique we could reliably characterize, in opacity units (OUs), the range of variation observed in freshly isolated strains as well as the remarkable heterogeneity found in many stock

Fig. 5. SDS-PAGE of outer-membrane proteins (a) and LPS (b) of: (a) strains 3083-O, 3083-T, MDO-12-C, MDO-12-B and MDO-12-A (lanes 1–5, respectively); and (b) MDO-12-A, MDO-12-B, MDO-12-C, 3083-T and 3083-O (lanes 1–5 respectively). Panel (b) has been digitally processed to enhance contrast.

Table 5. Survival of spontaneous variants of *V. cholerae* in normal saline

Bacteria were cultivated on medium containing 0.5% glycerol (+) or not (–), diluted to approximately 10^6 viable cells per ml of normal saline, incubated for 24 h, and dilution-plated. Results are shown as percentage survival (mean of duplicate determinations; NG, no growth from $10 \mu\text{l}$).

Strain	Glycerol	4 °C	25 °C	37 °C
MDO-12-A	–	NG	NG	NG
	+	2.0	31.0	27.0
-B	–	0.3	3.5	3.0
	+	5.0	41.0	39.0
-C	–	11.0	64.6	61.0
	+	55.0	95.0	93.0
3083-T	–	0.2	0.6	0.003
	+	0.8	3.0	3.0
-O	–	0.05	1.2	NG
	+	4.0	33.0	31.0

cultures. The apparent discrepancy between previous observations on colonial opacity (Lankford, 1960; Finkelstein & Gomez, 1963; Finkelstein & Punyashthiti, 1967) resided in the culture media used by the different investigators. The addition of glycerol tended to increase colonial opacity. Electron microscopic observations revealed that this also led to the production of intracellular inclusion bodies. Some of these stained with malachite green, which had been reported (Sun *et al.*, 1994) to selectively stain PHB. This was confirmed by biochemical assay which, like the electron microscopic observations, also showed that the PHB granules were consumed under crowded conditions and with ageing of the culture. The levels of PHB produced on glycerol medium strongly correlated with the opacity of the culture observed in the presence or absence of glycerol. In fact, examination of an extensive series of strains and colonial variants revealed a significant difference in PHB levels between strains with > 5 OUs and those with 5 OUs or less. The reason why glycerol enhances opacity is probably because of its place in intermediary metabolism which leads – through pyruvate, acetyl-CoA, acetoacetyl-CoA to 3-hydroxybutyryl-CoA and, by the enzyme PHB synthase – to PHB (Leaf *et al.*, 1996). PHB may also serve as a carbonaceous energy storage mechanism (Leaf *et al.*, 1996; Östling *et al.*, 1993). Actually in subsequent tests, pyruvate was even more effective than glycerol in enhancing opacity (D. K. Sengupta and others, unpublished). Why this should reflect opacity as seen in the absence of glycerol (and correspondingly low PHB levels) is not yet clear and remains an important question for further investigation. Perhaps translucency, in the presence or absence of glycerol, could be coupled to a defect in the ability to synthesize PHB.

Others have concluded that encapsulation is the primary determinant of opacity of *V. cholerae* O139 (Johnson *et*

al., 1994; Weintraub *et al.*, 1994). The present findings are consistent with the hypothesis that opacity is associated with encapsulation in O139 but indicates that other factors may also be involved. Studies with the MDO-12 series of variants indicate that although the degree of encapsulation varies in the appropriate order of opacity, the more translucent variants also express a characteristic 27 kDa outer-membrane protein, they had a different LOS structure when compared with the opaque variant, and they expressed different glycolytic pathways (MR/VP). Nevertheless, both the most opaque and the most translucent variants were equally virulent for infant rabbits. We have reported separately (Sengupta *et al.*, 1996) that antibody against the O139 capsule is protective in infant mice. Although the capsule may contribute to virulence it may not be completely essential (D. K. Sengupta and others, unpublished). Interestingly, a *TnphoA* insertional mutant of MDO-12-C, with no capsular polysaccharide (detectable by Western blotting with specific anticapsular serum), was more opaque than some encapsulated variants, and it was found to cause fluid accumulation in suckling mice although it adhered to the intestinal mucosa 100-fold less effectively than parent MDO-12-C (D. K. Sengupta and others, unpublished).

It is clear from the present work that the ability to produce PHB is proportional to opacity in freshly isolated and carefully preserved cultures whereas in older stock cultures and variants the relationship is less proportional but still significant. What benefit is it to the cholera vibrios to vary their PHB content? PHB is regarded as a carbon and energy reserve material in bacteria (Page & Knosp, 1989; Anderson & Dawes, 1990) and it enhances survival of some bacteria during nutrient starvation. The present study suggests that this may be the case in *V. cholerae*. Vibrios with higher PHB content were better able to survive nutrient deprivation. PHB levels fell with age of the culture and the degree of crowding, as did the content of electron-translucent inclusions of strain MDO-12-C. Further studies are needed to identify these electron-translucent granules. Similar inclusions were found in *V. harveyi* cells stained with malachite green (Sun *et al.*, 1994). Survivability of O139 strains may also be promoted by the degree of encapsulation.

For most of the century since the discovery of cholera vibrios by Koch, human beings were considered to be their sole reservoir in nature. However, older as well as more recent studies have shown that they may persist, for varying periods, in brackish estuaries in cholera-endemic areas, perhaps in association with plankton and shellfish (Pollitzer, 1959, pp. 546–547, 858–860; Colwell & Spira, 1992; Glass & Black, 1992; Blake, 1994; Colwell & Huq, 1994; Kaysner & Hill, 1994; Mintz *et al.*, 1994; Tauxe *et al.*, 1994), which can serve as vehicles of infection. Recognizing that it is extremely difficult to establish whether they are of human or environmental origin, it would be interesting indeed to determine whether persistence of cholera vibrios in the environment is associated with opacity and PHB

content. Is it possible that the demonstrated capability of variation of *V. cholerae* strains, with regard to opacity, PHB content, capsulation, OMPs, LPS, and glycolytic pathway, provides mechanisms of assuring their survival outside the warm and nutritious milieu of the human gut? In addition to simply serving as a carbon and energy reserve, PHB turnover has been demonstrated to direct the formation of dormant cells (Reusch & Sadoff, 1981). Colwell and her associates (Colwell & Spira, 1992; Colwell & Huq, 1994) have implied that true cholera vibrios can exist autochthonously in brackish water and estuarine systems in a 'viable but nonculturable state', potentially to emerge with pathogenic and epidemic potential. It would be of interest to evaluate whether PHB is involved.

Using *TnphoA* insertional mutagenesis, we are trying to ascertain which properties – PHB, capsule, outer-membrane proteins, LOS, and/or glycolytic pathway – are most directly responsible for – or associated with – opacity/translucency. Preliminary results thus far suggest that PHB content, translucency-associated outer-membrane proteins and glycolytic pathway are constant markers and the other properties may be variable (D. K. Sengupta and others, unpublished). It will be important to apply molecular genetics to determine whether these are independent variables defined by multiple separate genetic pathways or whether they are the pleiotropic result of single genetic regulatory events. Studies on effects of other physiological manipulations, such as nutritional alterations and growth temperature, on opacity/translucency may also be revealing.

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